AWARD NUMBER: **W81XWH-14-1-0515**

TITLE: SLCO2B1 and SLCO1B3 as New Targets for Enhancing Androgen Deprivation Therapy for Prostate Cancer

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REPORT DATE: **October 2015**

TYPE OF REPORT: **Annual report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual report	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

SLCO2B1 and SLCO1B3 as New Targets for Enhancing Androgen Deprivation Therapy for Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0515

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dana-Farber Cancer Institute
450 Brookline Ave
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We found the association between the *SLCO2B1* exonic SNP rs12422149 and time to progression (TTP) on ADT. We also found that the intronic SNP rs1077858 was associated with overall survival. SLCO2B1 expression in normal prostate tissue and in 22RV1 cells carrying the major allele of SNP rs1077858 was significantly lower than those carrying the risk allele. *In vitro*, we showed SLCO2B1 expression levels correlated with DHEAS uptake by PC cells. Our data shown that statin use at the time of ADT initiation was associated with a significantly longer TTP on ADT even after adjusting for known prognostic factors. Our *in vitro* findings that statins competitively reduce DHEAS uptake and thus, effectively decrease the available intratumoral androgen pool, affords a plausible mechanism to support the clinical observation of prolonged TTP in statin users.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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1, Introduction

We now appreciate that the androgen receptor (AR) remains a critical factor activated by residual androgens in castration resistant prostate cancer (CRPC) patients. The residual prostatic androgen in part or largely derived from uptake of circulating adrenal androgens. DHEA (dehydroepiandrosterone) and its sulfated form, DHEAS are secreted in large amounts by the adrenal cortex and are precursors for the production of T (testosterone) and DHT (dihydrotestosterone), the most potent androgens, in the tumor microenvironment. Genetic variations at two *SLCO* (Solute Carrier Organ Anion Transporter Family) genes (3 SNPs for SLCO2B1 and 2 SNPs for SLCO1B3) may alter androgen uptake affecting prostate cancer outcomes. SLCO2B1/SLCO1B3 exhibit higher expression levels in CRPC tumors, suggesting these genes are important in maintaining the CRPC phenotype. Statins and estrogens have been shown to use the SLCO2B1/SLCO1B3 transporters. Statins appear to have an effect on the progression to advanced prostate cancer. Estrogens have an antitumor effect in CRPC. We hypothesize that statins and estrogens might potentially block the uptake of androgen precursors from the tumor microenvironment via interactions with SLCO2B1/SLCO1B3. Therefore, elucidating the role and inhibiting the transport activity of SLCO2B1/SLCO1B3 in CRPC will may ultimately lead to improve outcomes of prostate cancer. We intend to demonstrate that statins/estrogens compete for DHEAS/T uptake through the SLCO2B1/SLCO1B3 transporters of in prostate cancer. Combining ADT and statins or inhibitors of SLCO2B1/SLCO1B3 may significantly enhance the efficacy of ADT and the overall survival. Our study will reveal mechanisms mediating the development of CRPC; enhance our understanding of molecular mechanisms underlying the impact of statins or estrogens on prostate cancer; and suggest new therapeutic targets potentially improving the efficacy of ADT.

2, Keywords

prostate cancer, androgen deprivation therapy (ADT), time to progression (TTP), overall survival (OS,) SLCO2B1, SNP, DHEAS, uptake, statins.

3, Accomplishments

► What were the major goals of the project?

Aim 1: We will determine the impact of statins and estrogens on the uptake of DHEAS or T through SLCO2B1 and SLCO1B3 and their effects on prostate cancer cell lines.

Aim 2: We will determine the impact of expression levels of SLCO2B1 and SLCO1B3 on prostate tumor progression to CRPC in the presence or absence of statins or estrogens in animal models.

Aim 3: We will determine the association between prostate cancer survival and the use of statins and tumor expression levels of SLCO2B1 and SLCO1B3 in men with different genotypes of SLCO2B1 and SLCO1B3 in the Health Professionals Follow-up Study and ADT prostate cancer cohorts.

Aim 4: We will identify and optimize small molecule inhibitors of SLCO2B1 and SLCO1B3 and test these inhibitors in cell based and animal models of prostate cancer.

► What was accomplished under these goals?

During the past year, we have studied the statin use at the time of initiation of androgen deprivation therapy (ADT) and time to progression (TTP) in patients with hormone-sensitive prostate cancer (Mentioned in **Aim 1**) and examined the association of *SLCO2B1* genotypes with TTP and overall survival (OS) in patients receiving ADT for prostate cancer (Mentioned in **Aim 3**).

Since statins utilize SLCO2B1 to enter cells, we hypothesized that they may compete with DHEAS uptake and that statin use might prolong TTP while on ADT (**Aim 1**). To determine if statins interfere with DHEAS uptake, we performed *in vitro* studies using prostate cancer cell lines. Next, we queried our institutional clinical database to assess for an association between statin use and TTP on ADT.

Using our IRB approved institutional clinical database (Prostate CRIS), we identified 1265 patients with hormone-sensitive PC who had been treated with ADT (with or without an antiandrogen) from January 1996 until November 2013. Patients were excluded if they had insufficient follow-up data on PSA after ADT administration (n=131) or if statin use status was unknown (N=208), which left 926 patients for this analysis. Clinicodemographic data was captured from the CRIS database. The electronic medical record was reviewed for dates of initiation and progression on ADT and whether patients were on statins at the time of ADT initiation. Progression was defined as a minimum of 3 PSA rises. Date of progression was defined as date of first PSA rise (nadir + $\geq$0.02 ng/ml) or radiologic progression. Patient and disease characteristics were summarized as frequencies or the median and range of values. Characteristics were compared between statin users and non-users using chi-square and Wilcoxon rank sum tests. The primary outcome variable was TTP on ADT, defined as the duration of time from ADT initiation to the date of disease progression or censored at the date of last follow-up visit in patients who were progression-free. The association between statin use and TTP on ADT was estimated from multivariable Cox regression to estimate hazard ratios (HR) and 95% confidence intervals (CI), adjusting for pre-defined prognostic factors: biopsy Gleason score, primary therapy type, use of prior ADT in conjunction with local therapy, metastatic status, and PSA at ADT initiation.

Patient and disease characteristics at diagnosis and at ADT initiation are detailed in **Table 1** of **Appendices Paper 1** (page 41). Statin users tended to have a lower median PSA both at

diagnosis (9.1 vs. 11.8) and at ADT initiation (10.3 vs. 12.5). Duration from diagnosis to ADT initiation was longer in statin patients (3.9 vs. 2.3 years). Users were more likely to have lower stage disease (56% vs. 44% T1 disease) and less likely to have *de novo* metastases (11% vs. 18%) or nodal involvement (5% vs. 10%) at diagnosis. Statin users were more likely to have undergone local therapy or to have received ADT as part of local therapy (33% vs. 26%). Patients on statins were less likely to have metastases at ADT initiation (53% versus 63%) (p values <0.05).

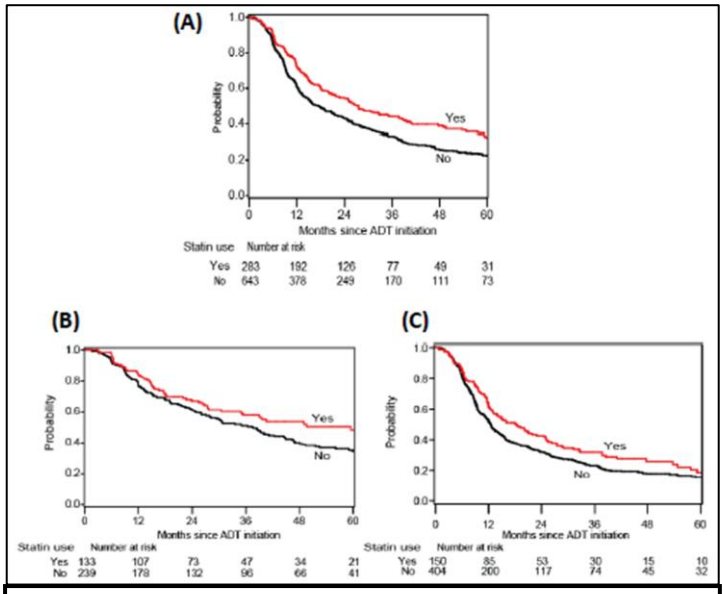

Figure 1: Kaplan Meier analysis of TTP on ADT according to statin use in all patients. Kaplan Meier plot of TTP on ADT according to statin use (A) In all patients (B) In patients with no visible radiographic metastasis but biochemical (PSA) failure (M0) and (C) in patients with metastasis (M1) at ADT initiation.

At the time of data capture, 70% (n=644) of patients had progressed on ADT by PSA. Median follow-up was 5.8 years (range: 0.1-15.9). Median TTP on ADT for all patients irrespective of statin use was 20.3 months (95% CI: 17.5, 23.6). Statin users at ADT initiation had a significantly longer median TTP on ADT (27.5 vs. 17.4 months, p=0.0005, **Fig 1A**). Since some but not all of the baseline characteristics favored more indolent disease in the statin users, we adjusted for these potential imbalances. Importantly, the association remained statistically significant after adjusting for pre-defined prognostic clinical factors including biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with localized therapy, metastatic status and PSA at initiation of ADT (adjusted HR=0.83, 95% CI: 0.69,0.99, **Table 2** of **Appendices Paper 1** (page 44)). When stratified by year of ADT initiation using 5-year increments, the association between statin use and TTP on ADT remained significant in the multivariable model (adjusted HR=0.83, 95% CI: 0.69,1.00). Moreover, the association between statin use and TTP was observed regardless of whether patients had radiographic evidence of metastatic disease compared to biochemical relapse only at ADT initiation (HR=0.79, 95% CI:0.58, 1.07 for M0; HR=0.84, 95% CI:0.67,1.06 for M1, p for interaction=0.72). (**Fig. 1B, 1C**)

In our ADT cohort of 926 patients, 283 (31%) were taking a statin at ADT initiation. After a median follow-up of 5.8 years, 644 patients (70%) had progressed on ADT. Median TTP on ADT was 20.3 months (95% CI: 18,24). Men on statins had a longer median TTP on ADT compared to non-users (27.5 vs. 17.4 months, p=0.0005). The association remained statistically significant after adjusting for pre-defined prognostic factors [adjusted HR=0.83 (p=0.039)]. The positive statin effect was observed for both patients with and without metastases. Therefore,

statin use at the time of ADT initiation was associated with a significantly longer TTP on ADT even after adjusting for known prognostic factors.

In vitro, we demonstrated that statins block DHEAS uptake by competitively binding to SLCO2B1. We examined the effect of four different statins (atorvastatin, fluvastatin, pravastatin and simvastatin) on DHEAS uptake in the androgen dependent LNCaP and the partially androgen dependent 22RV1 PC cell lines (**Fig. 2A**). DHEAS uptake in PC cell lines was concentration and time dependent (**Fig. 2B, 2C**). When incubated with DHEAS at a physiological concentration (2.5 µM) for 60 min, the 22RV1 cell line, which has a relatively high level of SLCO2B1 expression, displayed the most active DHEAS uptake of more than 300 pmol/mg compared to ~60 pmol/mg protein for LNCaP (**Fig. 2B**). 100 µM atorvastatin significantly decreased DHEAS influx by ~50% in both cell lines when cells were incubated with 2.5 µM DHEAS. Among the four statins we studied, pravastatin had the most significant inhibitory effect on DHEAS uptake in both cell lines, while a more prominent effect of simvastatin was shown for 22RV1 cells than that of LNCaP cells (**Fig. 2B**). However, 10 or even 100 µM atorvastatin or simvastatin was insufficient to inhibit DHEAS uptake in LNCaP, which has a relatively low level of SLCO2B1 expression when the concentration of 100 µM DHEAS was used. These results suggest, not surprisingly, that different statins compete with DHEAS for the same transporter but with varying efficiency and that this effect is cell line dependent.

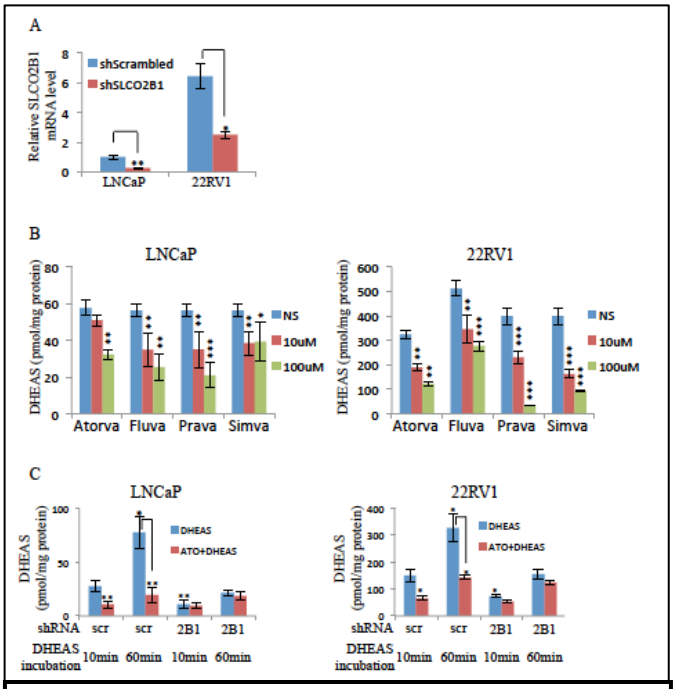

Figure 2: Statins inhibit DHEAS uptake by SLCO2B1 in PC cells. **A**. Relative mRNA levels in PC cells before and after SLCO2B1 is knocked down. **B.** DHEAS uptake in PC cells with 2.5 µM DHEAS and different concentrations of statins when incubated for 60 minutes. Statistical analysis was performed by comparing each condition with the DHEAS 2.5 µM and no statin state (NS) except when indicated. **C**. Depicts DHEAS uptake in PC cells before (scrambled shRNA (scr)) and after (shRNA 2B1) SLCO2B1 is knocked down when incubated with 2.5 µM DHEAS and 100 µM atorvastatin (ATO) for 10 and 60 minutes. Statistical analysis was performed by comparing each condition with scrambled shRNA (scr) after 10 min with DHEAS except when indicated.

To determine the dependence of DHEAS uptake on SLCO2B1, we constructed inducible SLCO2B1 deficient stable 22RV1 cell lines using the lentiviral derived tetracycline inducible shRNA knock down system. We were unable to establish an inducible SLCO2B1 deficient stable cell line in LNCaP, thus, we used a transient-inducible knock down of SLCO2B1 LNCaP cells in

this study (shRNA-SLCO2B1). After successfully knocking down SLCO2B1 (**Fig. 2A**), DHEAS uptake was substantially decreased to ~50% in 22RV1 and ~70% in LNCaP of that observed in control cells (cells transfected with scrambled shRNA) (**Fig. 2C**). These results indicate that SLCO2B1 plays an essential role in DHEAS import into PC cells (**Fig. 2C**). More importantly, knocking down SLCO2B1 abolished atorvastatin's inhibition of DHEAS uptake; further suggesting that atorvastatin competes with DHEAS for binding to their transporter, SLCO2B1.

To further support our hypothesis, we wanted to demonstrate that inhibition of cell growth was SLCO2B1 and DHEAS dependent. Thus, we examined the impact of atorvastatin, the most commonly used statin clinically, and DHEAS on tumor proliferation before and after SLCO2B1 was knocked down using the WST-1 assay.

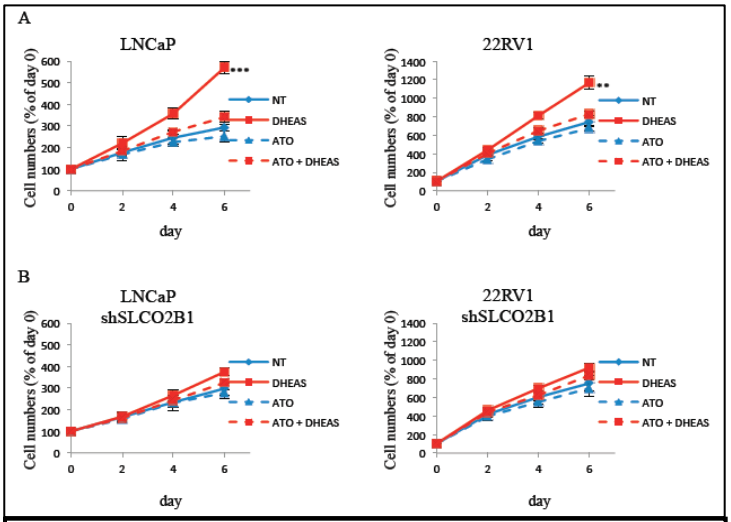

Figure 3. Atorvastatin (ATO) decreases DHEAS induced PC cell proliferation through SLCO2B1. Atorvastatin (ATO) decreases DHEAS induced PC cell proliferation by competing for influx through SLCO2B1. **A.** WST-1 assay of cell proliferation in PC cell lines. **B.** WST-1 assay of cell proliferation in PC cell lines after SLCO2B1is knocked down. For proliferation assays, cells were maintained in androgen depleted medium with doxycycline for 2 days followed by the addition of 80 nM DHEAS and 200 nM ATO to the culture medium for the indicated time. Relative cell numbers were calculated as percentages of the cell numbers at day 0 (100%).

2.5 μM (or a higher concentration) atorvastatin is known to inhibit LNCaP cell proliferation and induces autophagy. Since atorvastatin concentrations in patient serum range from 5-270 nM, we chose 200 nM of atorvastatin for our cell proliferation assay. 80 nM DHEAS significantly increased cell proliferation in LNCaP and 22RV1 lines that were maintained in androgen-depleted medium. Cell numbers nearly doubled by day 6 for LNCaP (**Fig. 3A**). At day 6, DHEAS induced a ~ 6-fold increase in LNCaP cell number, compared to an only ~3 fold increase in the absence of DHEAS ($p= 0.0003$). However, treatment with atorvastatin (200 nM) inhibited this DHEAS induced cell proliferation. Consistent with this finding, knocking down SLCO2B1 abolished DHEAS induced cell proliferation in LNCaP and 22RV1 (**Fig. 3B**). Furthermore, treatment with atorvastatin did not significantly inhibit the growth of SLCO2B1 knocked-down cells. Taken together, our data demonstrate that atorvastatin can efficiently block SLCO2B1-mediated DHEAS uptake and DHEAS induced cell growth in androgen dependent PC cell lines.

Our *in vitro* findings that statins competitively reduce DHEAS uptake and thus, effectively decrease the available intratumoral androgen pool, affords a plausible mechanism to support the clinical observation of prolonged TTP in statin users. This study was published in *JAMA oncology* (Please see the **Appendices Paper 1**, page 15).

In addition, we sought to validate the association of three previously demonstrated germline variants in *SLCO2B1* with TTP on ADT and to evaluate if these genetic variants were associated with OS for prostate cancer (partial of **Aim 3**). The three single nucleotide polymorphisms (SNPs) were genotyped in an independent validation cohort of 616 patients with PC treated with ADT at our institution from 1996 to 2013. Multivariable Cox regression adjusting for known prognostic factors estimated the association of genetic variants with TTP on ADT and OS. The expression of SLCO2B1 was examined in prostatectomy samples. The impact of SLCO2B1 expression level on uptake of DHEAS was evaluated in cell lines.

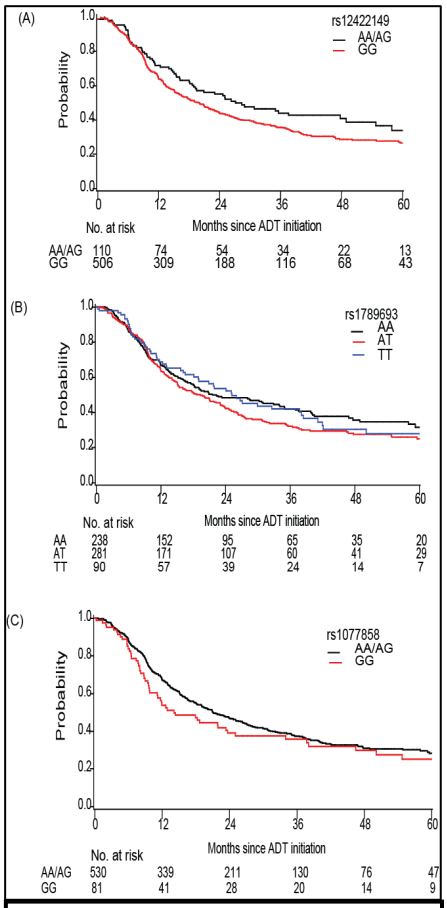

Figure 4. Kaplan-Meier curves of time to progression during ADT in the validation cohort, according to *SLCO2B1* genotypes (A) rs12422149, (B) rs1789693, (C) rs1077858.

In this study, the clinicodemographic, disease, and past treatment characteristics of the initial (N=478) and validation ADT cohort (n=616) are presented in **Supplementary Table 1** of **Appendices Paper 2** (page76). The two cohorts had similar disease characteristics at diagnosis and at ADT initiation. In both cohorts, approximately 70% of patients had received a local therapy (radical prostatectomy or radiation therapy) and nearly 60% had metastases at the time of ADT initiation. The validation cohort had a lower PSA at diagnosis (median 9.9 vs. 14 ng/ml), probably reflecting the more frequent use of PSA screening leading to an earlier diagnosis of PC in those patients. The validation cohort more frequently received intermittent ADT (35.9% vs 18.8%).

We first validated the Association of SLCO2B1 SNPs with TTP during ADT in the validation cohort (N=616). At the time of data retrieval, 66% (n=408) of patients in the validation cohort had progressed on ADT. The median TTP on ADT was 20.9 months (95% CI: 18.0, 24.0) and the median follow-up time was 4.2 years (range: 0.1, 16.3).

The exonic SNP, rs12422149, in *SLCO2B1* was associated with TTP on ADT in univariable analysis (median TTP: 27.2 (95% CI: 18.9,48.9) months for AA/AG and 20.0 (16.5,23.0) months for GG, *P* = .019) (**Fig 4** and **Table 1** of **Appendices Paper 2** (page 68)). In multivariable analyses adjusting for clinical factors, the association between the SNP and TTP remained significant (HR=1.31, 95% CI: 1.00, 1.72, *P*=.049). Thus, the association of rs12422149 with TTP on ADT observed in the initial cohort was validated such that PC patients carrying the major GG genotype exhibited a shorter TTP on ADT. While there was a trend toward an

association between rs1077858 with TTP on ADT, the two intronic SNPs were not validated (**Fig 4B, 4C**).

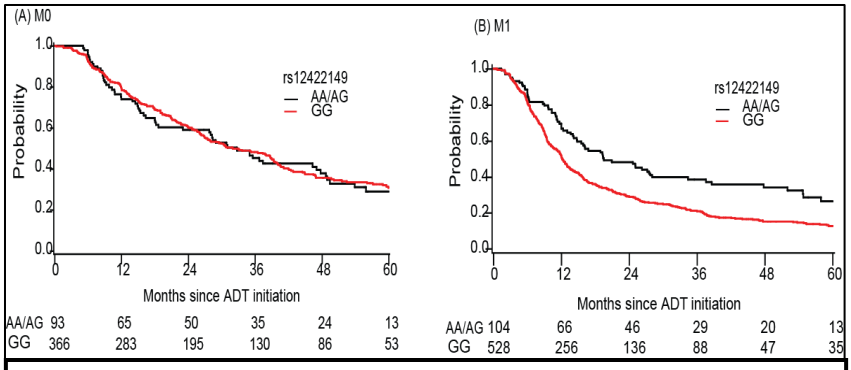

Figure 5. Kaplan-Meier curves of time to progression during ADT in all cohorts (initial plus validation cohort) according to rs12422149 genotypes as stratified by metastatic status (A) M0 versus (B) M1 at ADT initiation.

We then explored the correlation between the *SLCO2B1* SNPs and TTP in the combined initial and validation patients and stratified by metastatic disease status at time of ADT initiation, both of which had not been previously evaluated. In the combined cohort (n=1094), 74% of patients (n=811) had progressed on ADT. Median TTP on ADT was 18.9 months (95% CI: 16.5, 21.1). For the exonic rs12422149, we again found a significant association with TTP on ADT in the combined cohort (adjusted HR=1.33, 95%CI: 1.10-1.60, *P*= .003). When stratified by metastatic disease status, the association remained in the M1 population (adjusted HR=1.65, 95%: 1.28-2.12) but was not seen in the M0 population (adjusted HR=0.96, 95% CI: 0.72-1.27) (*P* interaction= .006, **Fig 5** and **Table 2** of **Appendices Paper 2** (page 69)). The association between the intronic SNP rs1077858 and TTP was of borderline significance (*P*= .075; *P*= .062 if AA and AG were combined). No association was found for rs1789693 and TTP on ADT in the combined cohort. Results were similarly negative in the M0 and M1 populations (*P* interaction >0.5). The associations with TTP on ADT were similar among patients with and without prior hormone treatments for the exonic SNP rs12422149. However, for the intronic SNP rs1077858, a significant association was observed only in patients without prior hormone treatment (adjusted HR=1.32 (1.06, 1.65)), but not in patients with prior hormone treatment (adjusted HR=0.87 (0.55, 1.37)).

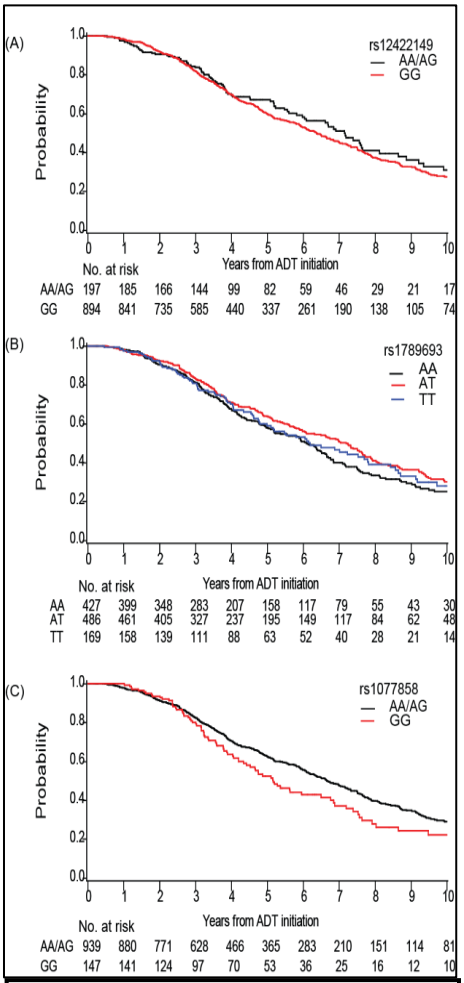

Fig 6. Kaplan-Meier curves of overall survival from ADT initiation in all patients (initial plus validation cohort), according to *SLCO2B1* genotypes (A) rs12422149, (B) rs1789693, (C) rs1077858.

Correlations with OS had not been explored in our prior work and thus we evaluated this endpoint in the combined cohort. Nearly half (49%, n=537/1094) of the patients had

died at the time of data collection. The median OS from ADT initiation was 6.5 years (95% CI: 6.0, 7.0) and the median follow-up time was 6.5 years (range 0.1, 16.3 years).

There was no statistically significant association with OS for the exonic SNP rs12422149 in either univariable or multivariable analyses (**Fig 6A** and **Table 3** of **Appendices Paper 2** (page 70)). In univariable analysis, there was no association between rs1789693 and OS from ADT initiation (*P*= .184, Bonferroni adjusted *P*=0.552), but patients carrying the minor allele (AT or TT) had longer OS in multivariable analysis (HR=0.81 and 0.78, respectively, *P*= .044) (**Fig 6B** and **Table 3 Appendices Paper 2** (page 70)). Importantly, we found that patients having the minor genotype GG in the intronic SNP rs1077858 had a shorter OS from ADT initiation in both univariable (*P*= .009, Bonferroni adjusted *P*=0.027) and multivariable analysis (adjusted HR=1.35(1.07, 1.71), *P*= .012) (**Fig 6C** and **Table 3 Appendices Paper 2** (page 70)). The median OS was decreased from 6.7 (95% CI: 6.2, 7.2) to 5.2 (95% CI: 4.3, 6.8) years with the AA/AG vs. GG rs1077858 genotypes respectively.

Given the fact that the intronic SNP rs1077858 was associated with OS, we studied the possible mechanism by which the SNP might affect OS from ADT initiation. Using 80 normal prostate tissue samples available in our tissue repository of patients known to have PC, we analyzed SLCO2B1 expression

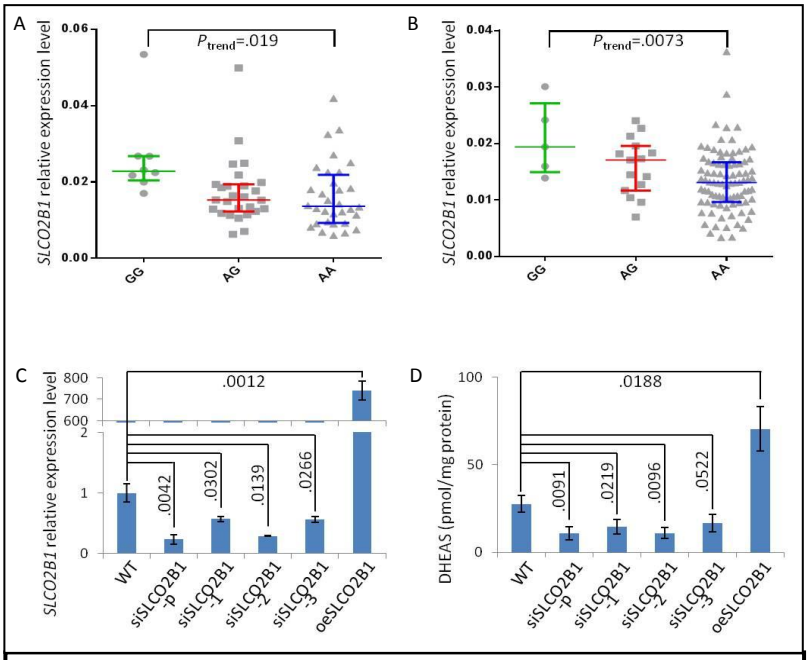

Figure 7. Impact of the intronic SNP rs1077858 on the expression of SLCO2B1 which affects the sulfated dehydroepiandrosterone (DHEAS) uptake. (A) Relative SLCO2B1 mRNA levels compared to GAPDH in normal prostate tissue in primary prostate cancer patients. Lines in the figure represent the median with interquartile range. (B) Relative SLCO2B1 mRNA levels compared to GAPDH in 22RV1 cells with different alleles after CRISPR reconstitution. Lines in the figure represent the median with interquartile range. (C) SLCO2B1 mRNA levels in mock transfected LNCaP cells and LNCaP cells transfected with different SLCO2B1 variants. Values represent the fold differences relative to those in mock transfected cells, which were set as 1.0.WT, LNCaP mock transfected with either siRNA control or pCMV6-XL4 vector; siSLCO2B1, LNCaP transiently transfected with siRNA targeting SLCO2B1 (-p represents the SMARTpool siRNA from GE Healthcare; -1, -2 and -3 represent three unique 27mer siRNAs, respectively.); oeSLCO2B1, LNCaP transiently transfected with pCMV6-XL4-SLCO2B1. Statistical analysis (unpaired t-test) was performed by comparing each condition with the WT condition. (D) DHEAS uptake in LNCaP cells. Cells were incubated with 2.5 µM DHEAS for 10 minutes after cells have been transfected with siRNA or pCMV6-XL4-SLCO2B1 for 3 days. All experiments were repeated in triplex. Statistical analysis (unpaired t-test) was performed by comparing each condition with the WT condition.

(**Fig 7A**). Results demonstrated that the different rs1077858 SNPs were associated with significantly different SLCO2B1 expression levels (P_{trend}= .019) with the highest SLCO2B1 expression in patients carrying the minor GG genotype. To prove that different rs1077858 SNPs impact the expression of SLCO2B1, we reconstituted the rs1077858 SNP variants using CRISPR in 22RV1 cells to convert the original A allele to the G allele (Supplementary data). The association between different rs1077858 SNPs and SLCO2B1 expression levels was significant (P_{trend}= .0073) and, once again, the GG genotype in 22RV1 cells corresponded to the highest SLCO2B1 expression level (**Fig 7B**). These results suggest that the association of rs1077858 on OS was possibly through changes in SLCO2B1 expression levels in these patients. As a result, alleles corresponding to higher SLCO2B1 expression may allow greater DHEAS uptake and thereby induce shorter OS in patients. To evaluate this hypothesis, we examined DHEAS uptake in a LNCaP PC cell line by altering the expression level of SLCO2B1 to mimic different rs1077858 allele conditions. After successfully knocking down or overexpressing SLCO2B1 (**Fig 7C**), we found that DHEAS uptake activity was dependent on the expression level of SLCO2B1 and that higher expression of SLCO2B1 transported more DHEAS into the cells (**Fig 7D**).Therefore, the association of the SNP rs1077858 with OS may be in part due to differential SLCO2B1 expression and consequent higher uptake of DHEAS and subsequent resistance to ADT, which in turn might contribute to shortened survival. This study was published in *JCO* (Please see the **Appendices Paper 2** (page 48)).

► What opportunities for training and professional development has the project provided?

Nothing to report.

► How were the results disseminated to communities of interest?

Nothing to report.

► What do you plan to do during the next reporting period to accomplish the goals?

- Determine the impact of estrogens on the uptake of DHEAS or T through SLCO2B1 and SLCO1B3 and their effects on prostate cancer cell lines.
- Determine the association between PCa survival and the use of statins and tumor expression levels of SLCO2B1 and SLCO1B3 in men with different genotypes of SLCO2B1 and SLCO1B3 in the Health Professionals Follow-up Study and ADT PCa cohorts.
- Determine the impact of expression levels of SLCO2B1 and SLCO1B3 on prostate tumor progression to CRPC in the presence or absence of statins or estrogens in animal models.

4, Impact

► What was the impact on the development of the principal discipline(s) of the project?

Almost all prostate cancers (PCa) depend on androgens for growth in its initial stages. We can suppress testicular androgen production with androgen deprivation therapy (ADT), which is the most effective and widely used therapy for PCa patients. However, residual androgen in patients still can active the androgen receptor (AR) and lead to castration resistant PCa (CRPC) which is usually lethal. DHEA (dehydroepiandrosterone) and its sulfated form, DHEAS are secreted in large amounts by the adrenal cortex and are precursors for the production of T (testosterone) and DHT (dihydrotestosterone), the most potent androgens. Therefore, blocking the uptake of adrenal androgens in the prostate could enhance the efficacy of ADT. SLCO2B1 and SLCO1B3 are two transporter genes involved in DHEAS and T uptake in the prostate. Evidence exists that SLCO2B1 and SLCO1B3 are expressed at higher levels in advanced PCa and the expression levels and genotypes of these two genes are correlated with the uptake activity of DHEAS and T and outcomes of PCa. Since statins and estrogens have been shown to use the SLCO2B1 and SLCO1B3 transporters and have an effect on the advanced prostate cancer, we hypothesize that statins and estrogens might work by blocking the uptake of androgens via interactions with SLCO2B1 and SLCO1B3. Therefore, illustrating the role and inhibiting the transport activity of SLCO2B1 and SLCO1B3 in CRPC will ultimately lead to improve outcomes of PCa. In this study, we will determine the mechanism if statins/estrogens compete for the DHEAS/T uptake through the SLCO2B1 and SLCO1B3 transporters. We will also design specific inhibitors of SLCO2B1 and SLCO1B3. A combination treatment with ADT and statins or inhibitors of SLCO2B1 and SLCO1B3 may significantly enhance the efficacy of ADT and outcomes in PCa patients.

New FDA approved agents such as abiraterone and enzalutamide successfully target the androgen pathway. But resistance in patients ultimately develops and adaptive mechanisms permitting the continued stimulation of AR by persistent ligand occurs. Novel therapeutic targets potentially improving the efficacy of ADT by further inhibition of residual androgens are needed. Based on our proposed study, we plan to develop SLCO2B1 and SLCO1B3 genotypes as biomarkers, but also improve the outcomes of PCa through inhibition of SLCO2B1 and SLCO1B3. Herein we will validate the basis of the putative benefit seen with statins and develop more potent inhibitors of this important adaptive pathway. If experimental results in our study develop as anticipated, we estimate that within 3-5 years, a clinically meaningful outcome will be achieved.

This study will reveal the mechanisms of CRPC development and enhance our understanding of molecular mechanisms underlying the impact of statins or estrogens on PCa progression. It will pave a new road to improve the efficacy of ADT by focusing on new therapeutic targets in PCa patients.

► What was the impact on other disciplines?

Nothing to report.

► What was the impact on technology transfer?

Nothing to report.

► What was the impact on society beyond science and technology?

Nothing to report.

5, Changes/Problems

► Changes in approach and reasons for change

Nothing to report.

► Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

► Changes that had a significant impact on expenditures

Nothing to report.

► Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

► Significant changes in use or care of human subjects

Nothing to report.

► Significant changes in use or care of vertebrate animals.

Nothing to report.

► Significant changes in use of biohazards and/or select agents

Nothing to report.

6, Products

► Publications, conference papers, and presentations

Harshman, L. C., Wang, X., Nakabayashi, M., Xie, W., Valenca, L., Werner, L., Yu, Y., Kantoff, A. M., Sweeney, C. J., Mucci, L. A., Pomerantz, M., Lee, G. S., and Kantoff, P. W. (2015) Statin

Use at the Time of Initiation of Androgen Deprivation Therapy and Time to Progression in Patients With Hormone-Sensitive Prostate Cancer, *JAMA oncology 1*, 495-504.

Wang, X., Harshman, L.C., Xie, W., Nakabayashi, M., Qu, F., Pomerantz, M., Lee, G.S., and Kantoff, P.W. (2015) Association of SLCO2B1 Genotypes with Time to Progression and Overall Survival in Patients Receiving Androgen Deprivation Therapy for Prostate Cancer, *Journal of Clinical Oncology*. Accepted.

Philip W. Kantoff, Xiaodong Wang, Wanling Xie, Mari Nakabayashi, Mark Pomerantz, Gwo-Shu Mary Lee. Genetic variants of the organic anion transporter SLCO2B1 as pharmacogenomic determinants of response to androgen deprivation therapy (ADT) for prostate cancer. Presentation, 2015 Genitourinary Cancers Symposium.

Lauren Christine Harshman, Xiaodong Wang, Mari Nakabayashi, Wanling Xie, Loana Bueno Valenca, Lillian Werner, Yongjiang Yu, Aaron Kantoff, Christopher Sweeney, Lorelei A. Mucci, Mark Pomerantz, Gwo-Shu Mary Lee, Philip W. Kantoff. Statin use at the time of initiation of androgen deprivation therapy and time to progression in patients with hormone-sensitive prostate cancer. Poster, 2015 ASCO Annual Meeting.

► Website(s) or other Internet site(s)

Statin drugs can delay prostate cancer progression in patients receiving androgen deprivation therapy: http://www.dana-farber.org/Newsroom/News-Releases/Statin-drugs-can-delay-prostate-cancer-progression-in-patients-receiving-androgen-deprivation-therapy-study-shows.aspx

Statins Associated with Longer Prostate Cancer Time to Progression During Androgen Deprivation Therapy: http://media.jamanetwork.com/news-item/statins-associated-with-longer-prostate-cancer-time-to-progression-during-androgen-deprivation-therapy/

Statin users have longer prostate cancer time to progression: http://www.healthylivingmagazine.us/Articles/9973/

Statin drugs can delay prostate cancer progression in patients receiving androgen deprivation therapy, study shows: http://www.sciencedaily.com/releases/2015/05/150507114004.htm

Concurrent Statin Use in ADT Patients Reduced All Cause, Prostate Cancer-Specific Deaths: http://www.onclive.com/publications/urologists-in-cancer-care/2015/August-2015/concurrent-statin-use-in-adt-patients-reduced-all-cause-prostate-cancer-specific-deaths#sthash.6BQd7wAk.dpuf

► Technologies or techniques

Nothing to report.

► Inventions, patent applications, and/or licenses

Nothing to report.

► Other Products

Nothing to report.

7, Participants & Other Collaborating Organizations

► What individuals have worked on the project?

Name:	*Philip Kantoff*
Project Role:	*PI*
Researcher Identifier:	
Nearest person month:	*12*
Contribution to Project:	*study design, preclinical studies, data analysis, data intepretation*
Funding Support:	*Dana-Farber Prostate Cancer SPORE P50CA090381*

Name:	*Xiaodong Wang*
Project Role:	*Key personal*
Researcher Identifier:	
Nearest person month:	*12*
Contribution to Project:	*preclinical studies, data collection, data analysis, data intepretation*
Funding Support:	

Name:	*Wanling Xie*
Project Role:	*Statistician*
Researcher Identifier:	
Nearest person month:	*4*
Contribution to Project:	*statistical analysis*
Funding Support:	

Name:	*Gwo-Shu Mary Lee*
Project Role:	Assistant to PI
Researcher Identifier:	
Nearest person month:	*12*

Contribution to Project:	studies, data collection, data analysis, data intepretation
Funding Support:	

Name:	*Lauren Harshman*
Project Role:	*Researcher*
Researcher Identifier:	
Nearest person month:	*6*
Contribution to Project:	*data analysis, data interpretation*
Funding Support:	

► Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

► What other organizations were involved as partners?

Nothing to report.

8, Special Reporting Requirements

► COLLABORATIVE AWARDS

Nothing to report.

► QUAD CHARTS

Nothing to report.

9, Appendices

Paper 1:

Statin use at the time of initiation of androgen deprivation therapy and time to progression in patients with hormone-sensitive prostate cancer.

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Key words: statins, prostate cancer, SLCO2B1, testosterone, DHEAS, androgen deprivation therapy, ADT

Abstract:

Importance: Statin use has been associated with improved prostate cancer outcomes. DHEAS is a precursor of testosterone and a substrate for SLCO2B1, an organic anionic transporter. We previously demonstrated that genetic variants of *SLCO2B1* correlated with time to progression (TTP) on androgen deprivation therapy (ADT).

Objective: Since statins utilize SLCO2B1 to enter cells, we hypothesized that they may compete with DHEAS uptake and that statin use might prolong TTP while on ADT.

Design: *In vitro* studies using prostate cancer cell lines. A retrospective analysis of prospectively collected data on 926 patients, who had received ADT from January 1996 until November 2013.

Setting: Academic, comprehensive cancer center.

Participants: Patients who had received ADT for biochemical or metastatic recurrence or *de novo* metastatic prostate cancer.

Main Outcomes and Measures: To determine if statins interfere with DHEAS uptake, we performed *in vitro* studies using prostate cancer cell lines. Next, we queried our institutional clinical database to assess for an association between statin use and TTP on ADT. TTP was estimated using multivariable Cox regression and adjusted for known prognostic factors.

Results: *In vitro,* we demonstrated that statins block DHEAS uptake by competitively binding to SLCO2B1. In our ADT cohort of 926 patients, 283 (31%) were taking a statin at ADT initiation. After a median follow-up of 5.8 years, 644 patients (70%) had progressed on ADT. Median TTP on ADT was 20.3 months (95% CI: 18,24). Men on statins had a longer median TTP on ADT compared to non-users (27.5 vs. 17.4 months, p=0.0005). The association remained statistically significant after adjusting for pre-defined prognostic factors [adjusted HR=0.83 (p=0.039)]. The positive statin effect was observed for both patients with and without metastases.

Conclusions and Relevance: Statin use at the time of ADT initiation was associated with a significantly longer TTP on ADT even after adjusting for known prognostic factors. Our *in vitro* findings that statins competitively reduce DHEAS uptake and thus, effectively decrease the available intratumoral androgen pool, affords a plausible mechanism to support the clinical observation of prolonged TTP in statin users.

Background

The organic anionic transporter, SLCO2B1 enables a variety of anticancer compounds and hormones to enter cells.[1] Amongst its substrates is the abundant adrenal androgen dehydroepiandrosterone sulfate (DHEAS), which is a precursor to more potent androgens, such as dihydroxytestosterone (DHT), which binds to the androgen receptor (AR) in normal and cancer cells. In prostate cancer (PC), expression of SLCO2B1 increases upon progression from hormone sensitive to castration-resistant disease.[2] Our group and others have previously demonstrated that genetic variants in *SLCO2B1* are associated with the durability of response to androgen deprivation therapy (ADT), due to varied efficiency of androgen influx into cells.[3,4]

Interestingly, statins are also substrates of SLCO2B1. Past work has generally shown an inverse association between statin use and incidence of PC as well as improved clinical outcomes.[5-8] Little is known about the impact of statin use and the durability of response to ADT, which is the cornerstone of treatment for metastatic hormone sensitive PC.[9] Given the fact that both DHEAS and statins are substrates for SLCO2B1, we first sought to determine if there was any interaction between statins and DHEAS influx by SLCO2B1 in PC cell lines. Then, using our prospectively collected institutional database, we evaluated the association between statin use and time to progression (TTP) among PC patients on ADT.

Methods

Cell lines and reagents

The hormone sensitive PC cell lines, LNCaP and 22RV1 were used in the study. LNCaP and 22RV1 cells were maintained in RPMI 1640 and supplemented with 10% FBS and antibiotics.

For the cell proliferation studies, all PC cells were cultured in Phenol-Red free RPMI 1640/10% charcoal-stripped FBS. 293T cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and antibiotics. All cell lines were regularly screened for mycoplasma (Sigma Venor GeM Mycoplasma Detection Kit). DHEAS was obtained from BioVender R&D Products. Atorvastatin was obtained from Santa Cruz Biotechnology, and fluvastatin, pravastatin and simvastatin were purchased from Selleckchem.

shRNA

We constructed an inducible shSLCO2B1 RNA expressing plasmid using the "all-in-one" pLKO-Tet-On lentiviral vector (see supplementary eMethods). Lentiviruses were packaged using 293T cells. The stable inducible shSLCO2B1-expressing cell lines were established by selection in puromycin. The efficiency of knocking down SLCO2B1 expression was assayed after induction with 1 ng/ml of doxycycline for 48 hours. The LNCaP and 22RV1 cell lines were transfected with scrambled shRNA, and these were used as a negative controls.

Quantitative RT-PCR

100 ngs total RNA was extracted from each PC cell line and analyzed by RT-PCR (see supplementary eMethods). All RT-PCR experiments were performed in triplicate.

DHEAS uptake assay

Cells were harvested in the PBS buffer and triple washed with incubation buffer (140 mM NaCl, 5 mM KCl, 1mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM D-glucose, and 12.5 mM

HEPES, pH 7.7). Aliquoted cells were incubated with each statin or DMSO (control) in incubation buffer at 37˚C for 10 minutes, and then treated with different DHEAS concentrations. At defined time points, the cells were triple washed with cold incubation buffer to stop DHEAS uptake. The cells were then lysed using 1% Triton X-100 solution in 1 × PBS on ice for 30 minutes. The total protein concentration was measured in the cleared cell lysate supernatant by protein BCA (bicinchoninic acid) assay (Thermo Scientific). The quantity of intracellular DHEAS was determined using the DHEAS ELISA kit (BioVendor, Candler, NC) and adjusted to the protein concentration of the cell lysate.

Cell proliferation assay

Cell proliferation was determined using the WST-1 assay (Roche, Indianapolis, IN). Briefly, control and shSLCO2B1-lentivirus infected cells were cultured in 96-well plates in the presence of doxycycline at a confluence of ~10% in androgen-depleted medium for 2 days followed by treatment with atorvastatin and/or DHEAS. Cell proliferation assays were carried out on different days after treatment. Each experiment was performed in triplicate.

Clinical cohort study

Using our IRB approved institutional clinical database (Prostate CRIS),[10] we identified 1265 patients with hormone-sensitive PC who had been treated with ADT (with or without an antiandrogen) from January 1996 until November 2013. Patients were excluded if they had insufficient follow-up data on PSA after ADT administration (n=131) or if statin use status was unknown (N=208), which left 926 patients for this analysis.

Clinicodemographic data was captured from the CRIS database. The electronic medical record was reviewed for dates of initiation and progression on ADT and whether patients were on statins at the time of ADT initiation. Progression was defined as a minimum of 3 PSA rises. Date of progression was defined as date of first PSA rise (nadir + $\geq$0.02 ng/ml) or radiologic progression.

Statistical analysis

Patient and disease characteristics were summarized as frequencies or the median and range of values. Characteristics were compared between statin users and non-users using chi-square and Wilcoxon rank sum tests. The primary outcome variable was TTP on ADT, defined as the duration of time from ADT initiation to the date of disease progression or censored at the date of last follow-up visit in patients who were progression-free. The association between statin use and TTP on ADT was estimated from multivariable Cox regression to estimate hazard ratios (HR) and 95% confidence intervals (CI), adjusting for pre-defined prognostic factors: biopsy Gleason score, primary therapy type, use of prior ADT in conjunction with local therapy, metastatic status, and PSA at ADT initiation.[11,12]

For *in vitro* studies, data was represented as means ± S.D. of at least 3 biological repeats. Comparison between two independent groups (or cell lines) was performed by a 2-tailed t test. $P < 0.05$ was considered statistically significant for all analyses.

Results

Inhibition of DHEAS uptake by atorvastatin is SLCO2B1 dependent

We examined the effect of four different statins (atorvastatin, fluvastatin, pravastatin and simvastatin) on DHEAS uptake in the androgen dependent LNCaP and the partially androgen dependent 22RV1 PC cell lines (Fig. 1A). DHEAS uptake in PC cell lines was concentration and time dependent (Fig. 1B, 1C, and eFig. 1). When incubated with DHEAS at a physiological concentration (2.5 μM) for 60 min, the 22RV1 cell line, which has a relatively high level of SLCO2B1 expression, displayed the most active DHEAS uptake of more than 300 pmol/mg compared to ~60 pmol/mg protein for LNCaP (Fig. 1B). 100 μM atorvastatin significantly decreased DHEAS influx by ~50% in both cell lines when cells were incubated with 2.5 μM DHEAS. Among the four statins we studied, pravastatin had the most significant inhibitory effect on DHEAS uptake in both cell lines, while a more prominent effect of simvastatin was shown for 22RV1 cells than that of LNCaP cells (Fig. 1B). However, 10 or even 100 μM atorvastatin or simvastatin was insufficient to inhibit DHEAS uptake in LNCaP, which has a relatively low level of SLCO2B1 expression when the concentration of 100 μM DHEAS was used (eFig.1). These results suggest, not surprisingly, that different statins compete with DHEAS for the same transporter but with varying efficiency and that this effect is cell line dependent.

To determine the dependence of DHEAS uptake on SLCO2B1, we constructed inducible SLCO2B1 deficient stable 22RV1 cell lines using the lentiviral derived tetracycline inducible shRNA knock down system. We were unable to establish an inducible SLCO2B1 deficient stable

cell line in LNCaP, thus, we used a transient-inducible knock down of SLCO2B1 LNCaP cells in this study (shRNA-SLCO2B1). After successfully knocking down SLCO2B1 (Fig. 1A), DHEAS uptake was substantially decreased to ~50% in 22RV1 and ~70% in LNCaP of that observed in control cells (cells transfected with scrambled shRNA) (Fig. 1C). These results indicate that SLCO2B1 plays an essential role in DHEAS import into PC cells (Fig. 1C). More importantly, knocking down SLCO2B1 abolished atorvastatin's inhibition of DHEAS uptake; further suggesting that atorvastatin competes with DHEAS for binding to their transporter, SLCO2B1.

Inhibition of SLCO2B1 mediated DHEAS uptake by atorvastatin decreases PC cell proliferation

To further support our hypothesis, we wanted to demonstrate that inhibition of cell growth was SLCO2B1 and DHEAS dependent. Thus, we examined the impact of atorvastatin, the most commonly used statin clinically, and DHEAS on tumor proliferation before and after SLCO2B1 was knocked down using the WST-1 assay.

2.5 μM (or a higher concentration) atorvastatin is known to inhibit LNCaP cell proliferation and induces autophagy.[13] Since atorvastatin concentrations in patient serum range from 5-270 nM,[14] we chose 200 nM of atorvastatin for our cell proliferation assay. 80 nM DHEAS significantly increased cell proliferation in LNCaP and 22RV1 lines that were maintained in androgen-depleted medium. Cell numbers nearly doubled by day 6 for LNCaP (Fig. 2A). At day 6, DHEAS induced a ~ 6-fold increase in LNCaP cell number, compared to an only ~3 fold increase in the absence of DHEAS ($p= 0.0003$). However, treatment with atorvastatin (200 nM) inhibited this DHEAS induced cell proliferation. Consistent with this finding, knocking down

SLCO2B1 abolished DHEAS induced cell proliferation in LNCaP and 22RV1 (Fig. 2B). Furthermore, treatment with atorvastatin did not significantly inhibit the growth of SLCO2B1 knocked-down cells. Taken together, our data demonstrate that atorvastatin can efficiently block SLCO2B1-mediated DHEAS uptake and DHEAS induced cell growth in androgen dependent PC cell lines. This mechanism may explain the observation that statin use may be associated improved clinical outcomes in PC and led us to pursue the current clinical study.

Clinical Cohort Study

Of the 926 patients included, 283 patients (30.6%) were taking a statin at ADT initiation. The majority (93%) of patients remained on a statin at the time of progression or at last follow-up. Statin use generally increased over time from 1996-2013 (eFig 2). Of the 643 non-users, 72 started a statin while on ADT; in these patients, median time from ADT initiation to initial statin use was 24 months (IQR 12 to ~48 months).

Patient and disease characteristics at diagnosis and at ADT initiation are detailed in Table 1. Statin users tended to have a lower median PSA both at diagnosis (9.1 vs. 11.8) and at ADT initiation (10.3 vs. 12.5). Duration from diagnosis to ADT initiation was longer in statin patients (3.9 vs. 2.3 years). Users were more likely to have lower stage disease (56% vs. 44% T1 disease) and less likely to have *de novo* metastases (11% vs. 18%) or nodal involvement (5% vs. 10%) at diagnosis. Statin users were more likely to have undergone local therapy or to have received ADT as part of local therapy (33% vs. 26%). Patients on statins were less likely to have metastases at ADT initiation (53% versus 63%) (p values <0.05, Table 1).

At the time of data capture, 70% (n=644) of patients had progressed on ADT by PSA. Median follow-up was 5.8 years (range: 0.1-15.9). Median TTP on ADT for all patients irrespective of statin use was 20.3 months (95% CI: 17.5, 23.6). Statin users at ADT initiation had a significantly longer median TTP on ADT (27.5 vs. 17.4 months, p=0.0005, Fig 3A). Since some but not all of the baseline characteristics favored more indolent disease in the statin users, we adjusted for these potential imbalances. Importantly, the association remained statistically significant after adjusting for pre-defined prognostic clinical factors including biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with localized therapy, metastatic status and PSA at initiation of ADT (adjusted HR=0.83, 95% CI: 0.69,0.99, Table 2).[12] When stratified by year of ADT initiation using 5-year increments, the association between statin use and TTP on ADT remained significant in the multivariable model (adjusted HR=0.83, 95% CI: 0.69,1.00). Moreover, the association between statin use and TTP was observed regardless of whether patients had radiographic evidence of metastatic disease compared to biochemical relapse only at ADT initiation (HR=0.79, 95% CI:0.58, 1.07 for M0; HR=0.84, 95% CI:0.67,1.06 for M1, p for interaction=0.72). (Fig. 3B, 3C)

Discussion

Progression to CRPC depends in part on the continued reliance of the tumor on AR signaling and residual androgens. In our preclinical studies, we demonstrated that DHEAS and various statins compete for binding to the transporter SLCO2B1 and that treatment with statins competitively inhibit DHEAS uptake. We demonstrated that the adrenal androgen DHEAS, an important precursor to DHT, stimulates PC cell proliferation and that a statin drug, atorvastatin, can

diminish DHEAS-stimulated proliferation. These findings drove us to query whether statin use might influence TTP on ADT in patients with hormone-sensitive PC. Given the vagaries of defining progression in PC, in our clinical study, we narrowly defined TTP by PSA alone, which allows us to use PSA as a pharmacodynamic endpoint of androgen action.

Statins are administered widely in the United States for their clinically meaningful lipid lowering properties. Most epidemiological studies have shown significant associations between statin use and decreased incidence of advanced prostate cancer, risk of recurrence after local treatment, mortality, and PSA levels relative to non-users.[5,6,15-17] Conversely, most cohort and case-controlled studies have shown no association between statins and overall prostate cancer risk.[7,8,18,19] To our knowledge, none have reported on the impact of statin use on TTP on ADT.

Given the biologic heterogeneity observed in prostate cancer, what becomes clear is that more important than overall incidence is the impact of statin use on the development of *lethal* prostate cancer.[20] A recent meta-analysis collated data from 27 observational studies encompassing nearly 2 million patients and assessed the association of statin use on the risk of developing PC.[5] The pooled analysis revealed a 7% reduction in the risk of developing any PC (RR 0.93, 95% CI 0.87-0.99, p=0.03). Seven of the studies specifically assessed the association between statin use and the risk of clinically significant or advanced PC. All but one showed a relative risk reduction ranging from 7-49% with statin use (RR: 0.51-0.93). As reviewed by Mucci and Stampher, there have been at least 5 additional published studies since 2012 that demonstrate an inverse association between statin use and prostate cancer mortality.[20]

Our study evaluated the impact of statin use in a more advanced patient population, all of whom had either biochemical or metastatic recurrence after local therapy or *de novo* metastatic disease for which they were started on ADT. We found that statin use at the time of ADT initiation was associated with a significant increase in TTP on ADT even after adjusting for established prognostic factors such as Gleason score at biopsy, type of primary therapy be it radical prostatectomy or radiation, use of ADT with the primary therapy, or presence of metastases and PSA at ADT initiation.[11,12]

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme critical in cholesterol biosynthesis.[21] Cholesterol is an essential component in the synthesis of steroid hormones such as the androgens that drive PC. Other purported mechanisms by which statins may exert their antitumor effects include inhibition of cell proliferation, inflammation, angiogenesis, invasion, and metastasis as well as induction of apoptosis and autophagy.[5,22-26] A reduced pool of cholesterol building blocks may stymie cancer growth as rapidly proliferating cells like tumor cells require high levels of available cholesterol.[23] Further, inhibition of HMG-CoA results in reduction of mevalonate and downstream farnesylpyrophosphate and geranylpyrophosphate, which are important in activating Ras and Rho.[21,27] Diminished Ras and Rho activity may inhibit cellular proliferation while stimulating apoptosis.[26,27]

Multiple preclinical models have assessed the direct effects of statins on prostate cancer. Zheng and colleagues evaluated atorvastatin and celecoxib in xenograft models.[28] They observed a reduction in PC cell growth even after castration and a delay in progression to androgen independence. *In vitro*, Murtola et al evaluated the effects of simvastatin using cell lines ranging

from normal prostate epithelium to resected primary tumors to more advanced CRPC-like cell lines such as LNCaP and VCaP.[27] They found that simvastatin inhibited early stage cell lines but not the more advanced CRPC-like subtypes.[5,27] While not directly assessing mechanism, meta-analysis of multiple randomized controlled trials and individual cohort studies in humans have shown that statins lower testosterone levels.[29,30]

Using PC cell lines, we found that statins and DHEAS compete for the same transporter, SLCO2B1, and that administration of statins competitively reduces uptake of DHEAS and subsequent tumor cell proliferation (atorvastatin). Thus, statins effectively decrease the available intratumoral androgen pool, which may enhance cancer control. These cell line findings provide a plausible mechanism to support our clinical observations of increased TTP on ADT in statin users.

An important limitation of our study is the retrospective nature in which we gathered data on statin use. As such, usage information was missing in 16% of the patients in our cohort. Several clinical factors significantly differed in statin users compared to non-users. At ADT initiation, patients on statins tended to have less advanced PC with less nodal or metastatic disease. In contrast, statin users were more likely to have received prior ADT with local therapy, which might have impacted the durability of their subsequent use. Nonetheless, we adjusted for these imbalances in our multivariate analysis. Another confounding variable, which we did not specifically address, was the contribution of different statins on TTP. It is conceivable that differences in statin potency and/or their pharmacokinetic properties[22] could have influenced the magnitude of the results.

Conclusion

We have shown that statins compete with DHEAS for influx by SLCO2B1, which may decrease the tumor's available androgen pool. Clinically, this may translate to improved cancer outcomes, which we observed in our institutional cohort of men receiving primary ADT. Even when controlled for known prognostic factors, men on statins had a significantly longer TTP on ADT than non-users. The mechanism through which statins exert their activity in PC is likely multifactorial including antiproliferative and proapoptotic effects but most plausibly is the reduction in the tumor's androgen stores through a combination of decreased availability of the cholesterol precursor required for *de novo* synthesis and decreased transport of existing precursor androgens like DHEAS via competitive binding of SLCO2B1. The widespread use of statins and their established safety profile make them attractive potential anticancer therapeutics as adjuvants to cytotoxic or androgen ablating therapies or as preventative agents. Ultimately, these results require prospective validation. Over 10 prospective trials are ongoing or maturing that will further characterize the role of statins as anticancer therapies in PC. (clinicaltrials.gov accessed 11/22/14).

Acknowledgements: Dr. Kantoff designed the studies and had full access to all of the data in the study. He takes responsibility for the integrity of the data and accuracy of the data analysis.

Lauren C. Harshman: literature search, data analysis, data interpretation, primary writing

Xiaodong Wang: literature search, preclinical studies, figure creation, data collection, data analysis, data intepretation, primary writing

Mari Nakabayashi: data collection and interpretation, manuscript review and critique

Wanling Xie: statistical analysis, figure creation, writing

Loana Valenca: data collection, manuscript review and critique

Lillian Werner: data collection, statistical analysis

Yongjiang Yu: preclinical studies, data collection

Aaron M. Kantoff: data interpretation, manuscript review and critique

Christopher Sweeney: data interpretation, manuscript review and critique

Lorelei A. Mucci: data interpretation, manuscript review and critique

Mark Pomerantz: data interpretation, manuscript review and critique

Gwo-Shu Mary Lee: preclinical studies, data collection, data analysis, data intepretation, manuscript review and critique

Philip W. Kantoff: study design, preclinical studies, data analysis, data intepretation, primary writing

Funding source: The research was funded by the Dana-Farber Prostate Cancer SPORE P50CA090381 and by the Department of Defense (DOD - W81XWH-14-1-0515)

Conflicts of Interest

AK serves as Vice President of Apple Tree Pharmaceuticals, which is an investor in Tokai Pharmaceuticals.

LH, XW, MN, WX, LV, LW, YY, CS, LM, GML, PK: none

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Figure Legend

Figure 1. Statins inhibit DHEAS uptake by SLCO2B1 in PC cells. **A**. Relative mRNA levels in PC cells before and after SLCO2B1 is knocked down. **B.** DHEAS uptake in PC cells with 2.5 μM DHEAS and different concentrations of statins when incubated for 60 minutes. Statistical analysis was performed by comparing each condition with the DHEAS 2.5 μM and no statin state (NS) except when indicated. **C**. Depicts DHEAS uptake in PC cells before (scrambled shRNA (scr)) and after (shRNA 2B1) SLCO2B1 is knocked down when incubated with 2.5 μM DHEAS and 100 μM atorvastatin (ATO) for 10 and 60 minutes. Statistical analysis was performed by comparing each condition with scrambled shRNA (scr) after 10 min with DHEAS except when indicated.

Figure 2. Atorvastatin (ATO) decreases DHEAS induced PC cell proliferation through SLCO2B1.

Atorvastatin (ATO) decreases DHEAS induced PC cell proliferation by competing for influx through SLCO2B1. **A.** WST-1 assay of cell proliferation in PC cell lines. **B.** WST-1 assay of cell proliferation in PC cell lines after SLCO2B1is knocked down. For proliferation assays, cells were maintained in androgen depleted medium with doxycycline for 2 days followed by the addition of 80 nM DHEAS and 200 nM ATO to the culture medium for the indicated time. Relative cell numbers were calculated as percentages of the cell numbers at day 0 (100%).

Figure 3: Kaplan Meier analysis of TTP on ADT according to statin use in all patients.

Kaplan Meier plot of TTP on ADT according to statin use (A) In all patients (B) In patients with no visible radiographic metastasis but biochemical (PSA) failure (M0) and (C) in patients with metastasis (M1) at ADT initiation. (A) Across the entire cohort, patients on statins at the time of ADT initiation had a significantly longer median TTP on ADT (27.5 vs. 17.4 months, p=0.0005). The association between statin use and TTP was observed regardless of whether patients had evidence of metastatic disease (C) compared to biochemical relapse (B) only at ADT initiation (HR=0.79, 95% CI:0.58, 1.07 for M0; HR=0.84, 95% CI:0.67,1.06 for M1, p for interaction=0.72).

Supplementary Figures:

eFigure 1: The impact of statins on DHEAS uptake by prostate cancer cells.

DHEAS uptake in PC cells with 100 μM DHEAS and different concentrations of statins when incubated for 60 minutes. Statistical analysis was performed by comparing each condition with the DHEAS 2.5 μM and no statin state (NS).

eFigure 2: Statin use by year of ADT initiation. Statin use tended to increase over time with significantly more users after 2006 compared to prior to 2006.

Figure 1: Statins inhibit DHEAS uptake by SLCO2B1 in prostate cancer cells.

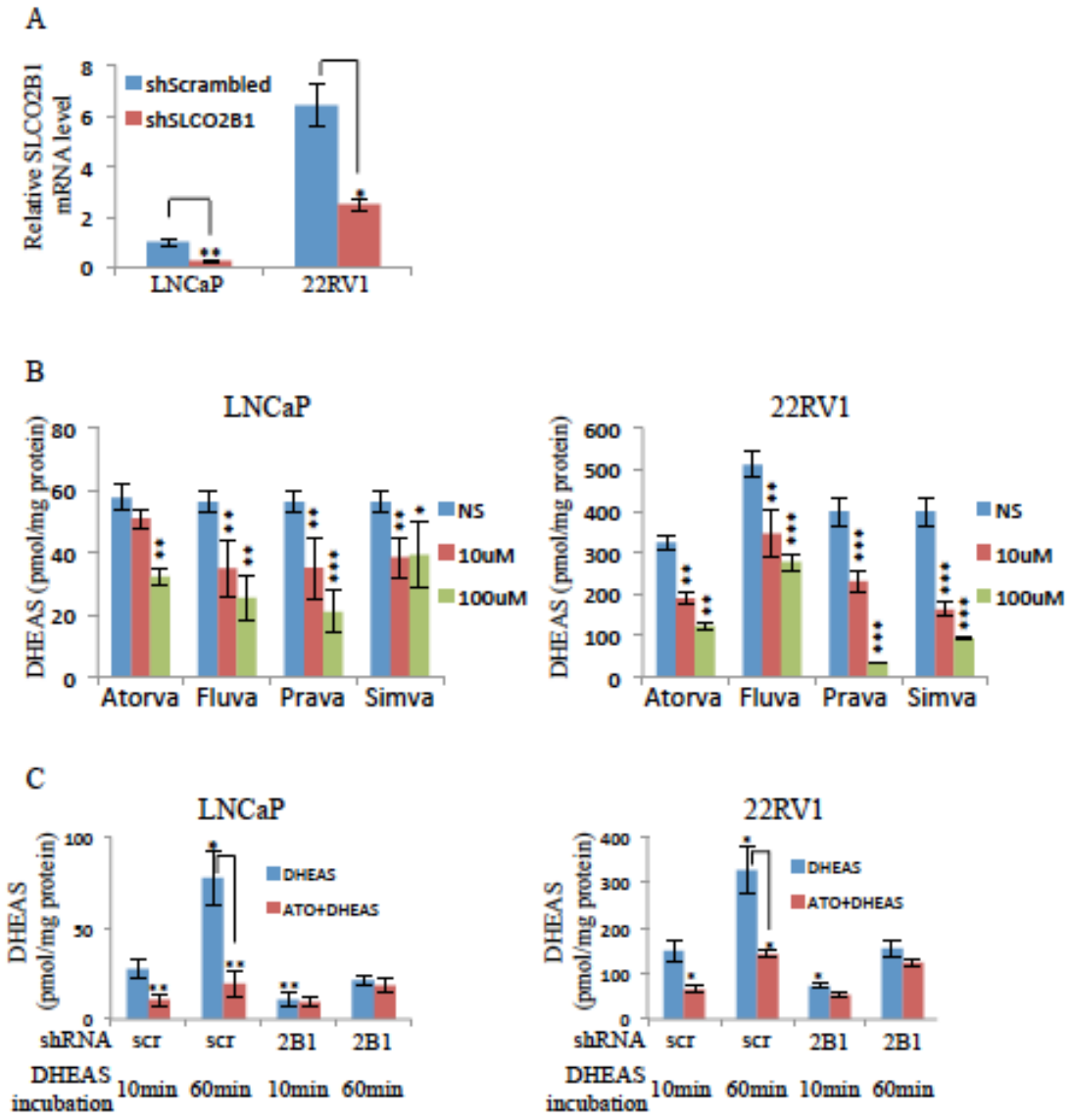


Figure 2: Atorvastatin (ATO) decreases DHEAS induced PC cell proliferation through SLCO2B1.

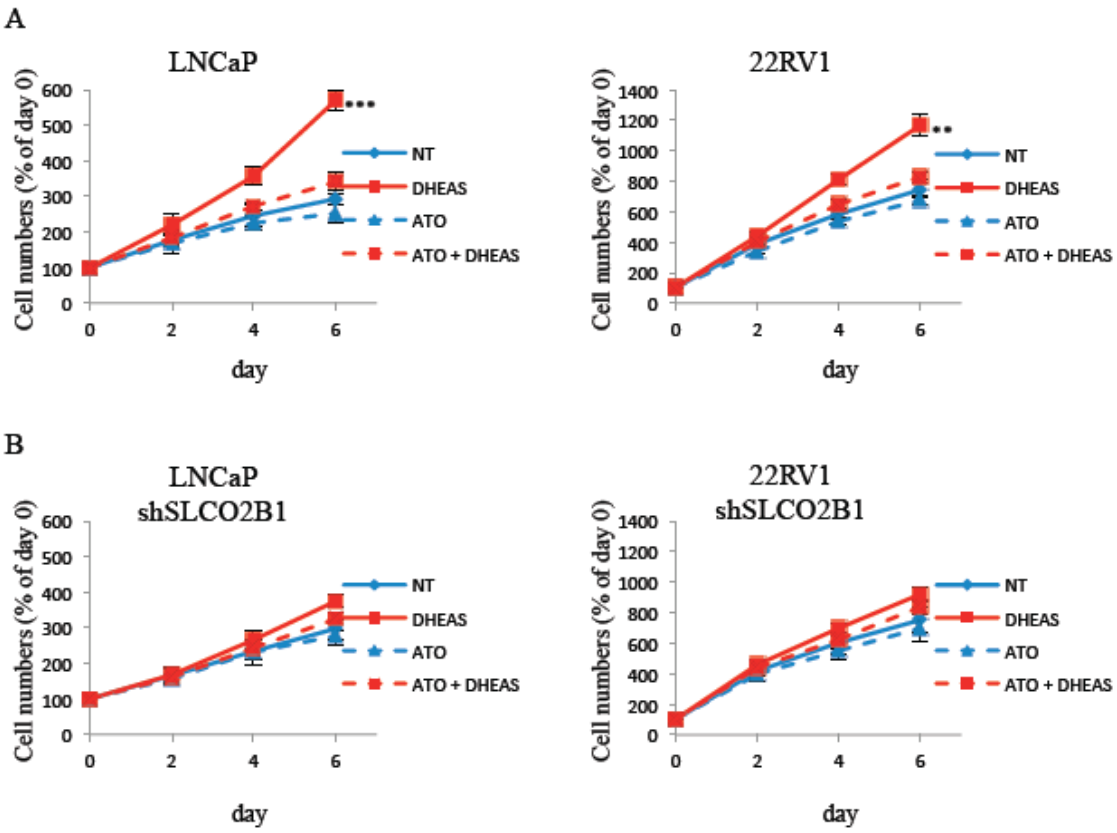


Figure 3: Kaplan Meier analysis of TTP on ADT according to statin use in all patients. Kaplan Meier plot of TTP on ADT according to statin use (A) In all patients (B) In patients with no visible radiographic metastasis but biochemical (PSA) failure (M0) and (C) in patients with metastasis (M1) at ADT initiation.

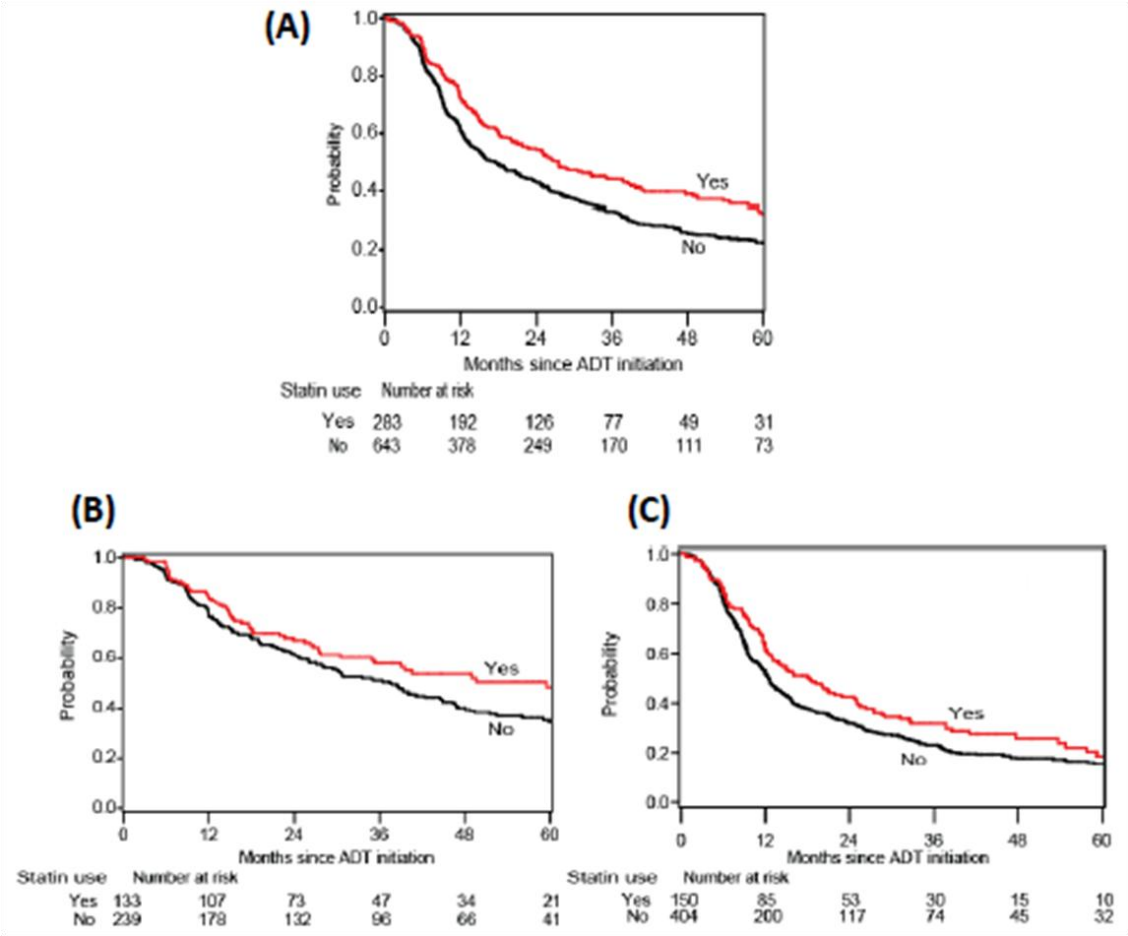


Table 1 Patient and disease characteristics at diagnosis and at ADT initiation by statin use in the GELB cohort (N=926)

	All		Statin Use				
			No (n=643)		Yes (n=283		
	N	%/Range	N	%/Range	N	%/Range	p-value
At Diagnosis							
Age, years (median, IQR)	881	61(55,67)	616	60(55,66)	265	62(56,67)	0.018
PSA, ng/mL (median, IQR)	813	10.7(6,29)	570	11.8(6.3,40)	243	9.1(5.5,17)	0.0003
Clinical T stage							
T1	442	47.7	283	**44.0**	159	**56.2**	0.005
T2	206	22.2	149	**23.2**	57	**20.1**	
T3-4	48	5.2	38	**5.9**	10	**3.5**	
Tx/Unknown	230	24.9	173	**26.9**	57	**20.1**	
Clinical M stage							
M0	338	36.5	235	**36.5**	103	**36.4**	0.013
M1	148	16.0	117	**18.2**	31	**11.0**	
Mx/Unknown	440	47.5	291	**45.3**	149	**52.7**	
Clinical N stage							
N0	337	36.4	235	**36.5**	102	**36.0**	0.031
N1	78	8.4	64	**10.0**	14	**5.0**	
Nx/Unknown	511	55.2	344	**53.5**	167	**59.0**	
Biopsy Gleason Score							
≤6	134	14.5	85	**13.2**	49	**17.3**	0.107
7	310	33.5	207	**32.2**	103	**36.4**	

	All		Statin Use				
			No (n=643)		Yes (n=283		
	N	%/Range	N	%/Range	N	%/Range	p-value
At Diagnosis							
Age, years (median, IQR)	881	61(55,67)	616	60(55,66)	265	62(56,67)	0.018
PSA, ng/mL (median, IQR)	813	10.7(6,29)	570	11.8(6.3,40)	243	9.1(5.5,17)	0.0003
≥8	376	40.6	272	**42.3**	104	**36.7**	
Unknown	106	11.4	79	**12.3**	27	**9.5**	
Type of local therapy							
RP +/-RT	388	41.9	268	**41.7**	120	**42.4**	<.0001
RT only/other	285	30.8	172	**26.7**	113	**39.9**	
None	253	27.3	203	**31.6**	50	**17.7**	
ADT as part of local therapy							
No	668	72.1	478	**74.3**	190	**67.1**	0.024
Yes	258	27.9	165	**25.7**	93	**32.9**	
At ADT Initiation							
PSA, ng/mL (median, IQR)	849	11.8(4.4,45)	580	12.5(4.4,59.1)	269	10.3(4.6,28.2)	0.038
Years from diagnosis to ADT initiation (median, IQR)	881	2.88(0.16,6.1)	616	2.33(0.1,5.6)	265	3.85(1.2,7.8)	<.0001
Metastases							
No	372	40.2	239	**37.2**	133	**47.0**	0.005
Yes	554	59.8	404	**62.8**	150	**53.0**	

	All		Statin Use				
			No (n=643)		Yes (n=283		
	N	%/Range	N	%/Range	N	%/Range	p-value
At Diagnosis							
Age, years (median, IQR)	881	61(55,67)	616	60(55,66)	265	62(56,67)	0.018
PSA, ng/mL (median, IQR)	813	10.7(6,29)	570	11.8(6.3,40)	243	9.1(5.5,17)	0.0003
Concomitant use, prior to progression, of							
5-alpha reductase inhibitor	32	3.5	25	**3.9**	7	**2.5**	0.278
Antiandrogen	658	71.1	459	**71.4**	199	**70.3**	0.742
Prior chemotherapy	72	7.8	40	**6.2**	32	**11.3**	0.008

Table 2: Association of statin use with TTP on ADT

	Statin users (N=283)	Non users (N=643)	P value
N (%) events	164(58)	480(75)	
Median TTP (95% CI), months	27.5 (21.1,37.7)	17.4 (14.9,21.1)	0.0005
Univariable HR (95% CI)	0.73 (0.61,0.87)	Reference	0.0005
Multivariable 1 HR*	0.83 (0.69,0.99)	Reference	0.0392
Multivariable 2 HR **	0.83 (0.69,1.00)	Reference	0.0499

*The multivariable model 1 was adjusted for biopsy Gleason score (<7, 7, >7), type of primary therapy, use of prior ADT in conjunction with local therapy, metastatic status and PSA at initiation of ADT (<10,10~20,≥20ng/mL). An unknown group was included if there were missing values for a factor.

**The multivariable model 2 was a stratified Cox regression, adjusting for the same variables as in model 1 and also stratified by year of ADT initiation using 5-year increments.

Supplementary Data

Supplementary Methods:

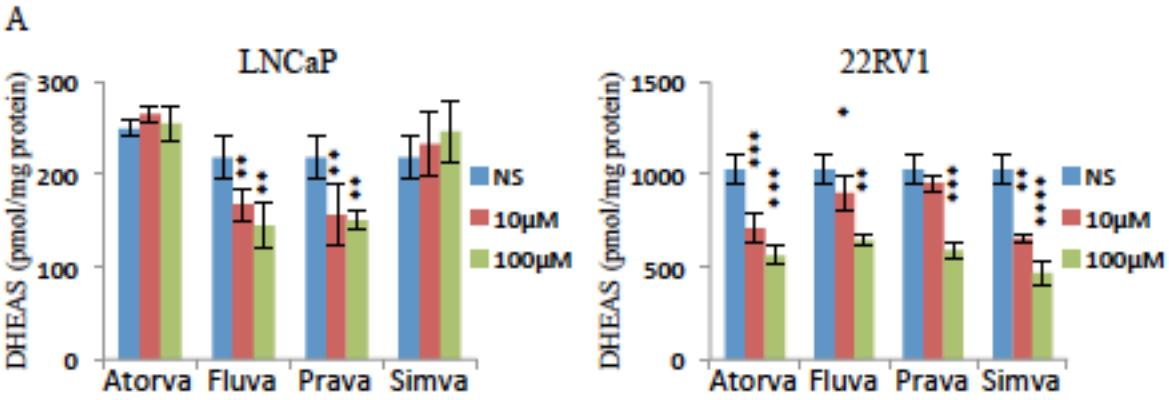
shRNA

We constructed the inducible shSLCO2B1 RNA expressing plasmid using the "all-in-one" pLKO-Tet-On lentiviral vector. The sequence of SLCO2B1 shRNA oligos: forward 5'-ccggGAGGAGAGGGTTTGCTAATCTctcgagAGATTAGCAAACCCTCTCCTCttttt-3' and reverse 5'-aattaaaaGAGGAGAGGGTTTGCTAATCTctcgagAGATTAGCAAACCCTCTCCTC-3'.

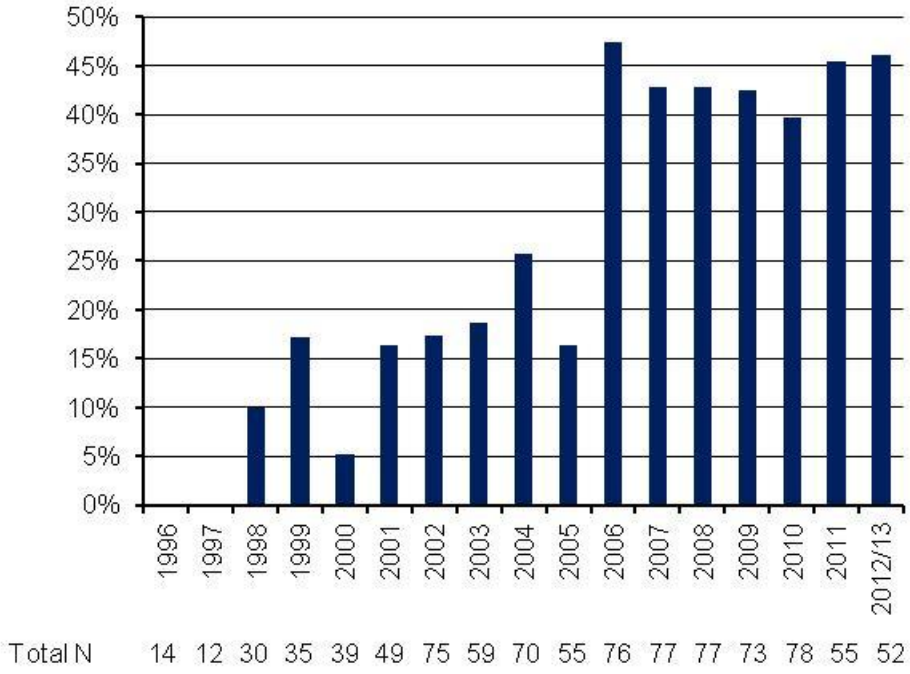
Quantitative RT-PCR

The following primers were used to measure the targeted gene expression level: SLCO2B1, forward 5'-GTTTCGGCGAAAGGTCTTAGCAG-3', reverse 5'-CCATCCTGCTTCTTCGTGGACT-3' and GAPDH, forward 5'-CAGCCTCAAGATCATCAGCA-3', reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'. The primer pair efficiencies for SLCO2B1 and GAPDH were calculated according to the linear regression of the log serial (8 ×) dilution factor versus the correspondent threshold cycle (Ct) values. The slope of the regression line was used to calculate the primer pair efficiencies (E), i.e., $E = 10^{(-1/slope)}$. The primer pairs with efficiencies close to 2 were used.

Supplementary Figure 1: The impact of statins on DHEAS uptake by prostate cancer cells.



Supplementary Figure 2: Statin use by year of ADT initiation



Paper 2:

Association of *SLCO2B1* Genotypes with Time to Progression and Overall Survival in Patients Receiving Androgen Deprivation Therapy for Prostate Cancer

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

Purpose

We sought to validate the association of three previously demonstrated germline variants in *SLCO2B1* with time to progression (TTP) on androgen deprivation therapy (ADT) and to evaluate if these genetic variants were associated with overall survival (OS) for prostate cancer (PC).

Patients and Methods

The three single nucleotide polymorphisms (SNPs) were genotyped in an independent validation cohort of 616 patients with PC treated with ADT at our institution from 1996 to 2013. Multivariable Cox regression adjusting for known prognostic factors estimated the association of genetic variants with TTP on ADT and OS. The expression of SLCO2B1 was examined in prostatectomy samples. The impact of SLCO2B1 expression level on uptake of DHEAS was evaluated in cell lines.

Results

The association between the exonic SNP rs12422149 and TTP on ADT was confirmed in univariable (*P*=.019) and multivariable (adjusted HR= 1.31, 95% CI: (1.00, 1.72) for GG vs. AA/AG, *P*=.049) analyses. Since OS had not been previously evaluated, we examined the association in the combined initial and validation cohorts (n=1094). The intronic SNP rs1077858 was associated with OS in both univariable (*P*=.009, Bonferroni adjusted *P*=.027) and multivariable (adjusted HR= 1.35, 95% CI: (1.07, 1.71) for GG vs. AA/AG, *P*=.012) analyses. SLCO2B1 expression in normal prostate tissue and in 22RV1 cells carrying the major allele of SNP rs1077858 was significantly lower than those carrying the risk allele. *In vitro*, we showed SLCO2B1 expression levels correlated with DHEAS uptake by PC cells.

Conclusion

The association of the SNP rs1077858 with OS may be in part due to differential SLCO2B1 expression and consequent higher uptake of DHEAS and subsequent resistance to ADT, which in turn might contribute to shortened survival.

INTRODUCTION

Androgen deprivation therapy (ADT) is the primary treatment for newly diagnosed metastatic prostate cancer (PC) by suppressing testicular androgen production and diminishing AR activation.[1,2] Most patients will eventually progress to castration resistant PC (CRPC) which is generally lethal.[3-6] The variability of time to castration-resistant disease may be partly due to inherited factors that result in increased uptake or utilization of available circulating androgens.

Despite treatment with ADT, persistent AR activation stimulated by residual androgen remains an integral factor that drives progression to castration resistance.[7,8] These residual androgens are derived either from increased *de novo* androgen synthesis or by uptake and conversion of circulating adrenal androgens.[6,9] One such adrenal androgen, dehydroepiandrosterone (DHEA) and its sulfated form, DHEAS are secreted in large amounts by the adrenal cortex. In the tumor microenvironment, DHEAS is converted to testosterone, and subsequently to dihydrotestosterone (DHT), which is the primary androgen utilized by PC cells.[10-12] Even after ADT, in humans, DHEAS persists at a high concentration in the blood at a range of 1-20 μM.[13-15]

How patients utilize available DHEAS may impact their response to ADT. The super family of organic anion transporting polypeptides (OATPs) encoded by the Solute Carrier Organ Anion Transporter Family (*SLCO*) genes, mediates the sodium-independent uptake of a wide variety of endogenous compounds and drugs into cells.[16,17] *SLCO2B1*, one of the *SLCO* gene family members, mediates the transport of endogenous steroid conjugates, such as DHEAS,[17-20] and is expressed in a broad range of tissues including the prostate. CRPC tumors exhibit higher SLCO2B1 expression than localized PC primary tumors.[21] Epidemiologic studies have demonstrated that sequence variations of *SLCO2B1* can have a significant impact on the time to

progression (TTP) to CRPC or on PC survival.[21-23] In our previous study, we genotyped 18 single nucleotide polymorphisms (SNPs) of *SLCO2B1* in a cohort of 538 patients with PC treated with ADT.[22] Three SNPs were associated with TTP on ADT ($p<0.05$): an exonic SNP rs12422149G>A and two intronic SNPs: rs1789693A>T and rs1077858A>G. The differences in median TTP for each of those three identified SNPs were approximately 10 months.

In this report, we attempted to validate whether these three *SLCO2B1* SNPs were associated with TTP in patients on ADT in an independent validation cohort. In addition, we examined the association of *SLCO2B1* SNPs with TTP and overall survival (OS) in the combined initial and validation cohorts as well as assessed the impact of metastatic disease status at ADT initiation.

PATIENTS AND METHODS

All cohorts were identified from our established, institutional Prostate Clinical Research Information System (CRIS) database.[24] Patients with biochemical recurrence (M0) or radiologically evident metastatic disease (M1) who had received ADT for hormone sensitive prostate cancer were identified. We started to register patients to CRIS in 2001 and all patients were consented to an IRB-approved protocol that collects clinical and genomic data. For the validation part, we used a cohort of 616 patients who had not been previously analyzed (validation cohort). For other analyses, we used the combined cohorts (N=1094), which also include 478 patients from the initial cohort[22]. The clinical data collection as well as the detailed patient characteristics are described in the Supplemental Data and have been previously described.[22]

Statistical methods were described in the Supplemental Data. Details of SNP genotyping, plasmid preparation, cell cultures, transfections, DHEAS uptake assays were described in the previous study.[22]

RESULTS

Patient Characteristics

The clinicodemographic, disease, and past treatment characteristics of the initial (N=478) and validation ADT cohort (n=616) are presented in Supplementary Table 1. The two cohorts had similar disease characteristics at diagnosis and at ADT initiation. In both cohorts, approximately 70% of patients had received a local therapy (radical prostatectomy or radiation therapy) and nearly 60% had metastases at the time of ADT initiation. The validation cohort had a lower PSA at diagnosis (median 9.9 vs. 14 ng/ml), probably reflecting the more frequent use of PSA screening leading to an earlier diagnosis of PC in those patients. The validation cohort more frequently received intermittent ADT (35.9% vs 18.8%).

Validation of the Association of SLCO2B1 SNPs with TTP during ADT in the validation cohort (N=616)

At the time of data retrieval, 66% (n=408) of patients in the validation cohort had progressed on ADT. The median TTP on ADT was 20.9 months (95% CI: 18.0, 24.0) and the median follow-up time was 4.2 years (range: 0.1, 16.3).

The exonic SNP, rs12422149, in *SLCO2B1* was associated with TTP on ADT in univariable analysis (median TTP: 27.2 (95% CI: 18.9,48.9) months for AA/AG and 20.0 (16.5,23.0) months for GG, P = .019)(Table 1 and Supplementary Fig 1A). In multivariable analyses adjusting for

clinical factors, the association between the SNP and TTP remained significant (HR=1.31, 95% CI: 1.00, 1.72, *P*=.049). Thus, the association of rs12422149 with TTP on ADT observed in the initial cohort was validated such that PC patients carrying the major GG genotype exhibited a shorter TTP on ADT. While there was a trend toward an association between rs1077858 with TTP on ADT, the two intronic SNPs were not validated (Table 1 and Supplementary Fig 1B, 1C).

Association of SLCO2B1 SNPs with TTP during ADT in the Combined ADT Cohorts Based on Presence of Metastases at ADT Initiation and prior ADT use.

Radiographically evident metastatic PC (M1) carries a worse prognosis than biochemically recurrent disease (PSA only or M0). Thus, we explored the correlation between the *SLCO2B1* SNPs and TTP in the combined initial and validation patients and stratified by metastatic disease status at time of ADT initiation, both of which had not been previously evaluated. In the combined cohort (n=1094), 74% of patients (n=811) had progressed on ADT. Median TTP on ADT was 18.9 months (95% CI: 16.5, 21.1).

For the exonic rs12422149, we again found a significant association with TTP on ADT in the combined cohort (adjusted HR=1.33, 95%CI: 1.10-1.60, *P*= .003). When stratified by metastatic disease status, the association remained in the M1 population (adjusted HR=1.65, 95%: 1.28-2.12) but was not seen in the M0 population (adjusted HR=0.96, 95% CI: 0.72-1.27) (*P* interaction= .006, Table 2 and Supplementary Fig 2). The association between the intronic SNP rs1077858 and TTP was of borderline significance (*P*= .075; *P*= .062 if AA and AG were combined). No association was found for rs1789693 and TTP on ADT in the combined cohort. Results were similarly negative in the M0 and M1 populations (*P* interaction >0.5, Table 2 and Supplementary Fig 2).

The associations with TTP on ADT were similar among patients with and without prior hormone treatments for the exonic SNP rs12422149. However, for the intronic SNP rs1077858, a significant association was observed only in patients without prior hormone treatment (adjusted HR=1.32 (1.06, 1.65)), but not in patients with prior hormone treatment (adjusted HR=0.87 (0.55, 1.37)) (Supplementary Table 2).

Association of SLCO2B1 SNPs with OS in the Combined ADT Cohorts

Correlations with OS had not been explored in our prior work and thus we evaluated this endpoint in the combined cohort. Nearly half (49%, n=537/1094) of the patients had died at the time of data collection. The median OS from ADT initiation was 6.5 years (95% CI: 6.0, 7.0) and the median follow-up time was 6.5 years (range 0.1, 16.3 years).

There was no statistically significant association with OS for the exonic SNP rs12422149 in either univariable or multivariable analyses (Table 3 and Supplementary Fig 3A). In univariable analysis, there was no association between rs1789693 and OS from ADT initiation (*P*= .184, Bonferroni adjusted *P*=0.552), but patients carrying the minor allele (AT or TT) had longer OS in multivariable analysis (HR=0.81 and 0.78, respectively, *P*= .044) (Table 3 and Supplementary Fig 3B). Importantly, we found that patients having the minor genotype GG in the intronic SNP rs1077858 had a shorter OS from ADT initiation in both univariable (*P*= .009, Bonferroni adjusted *P*=0.027) and multivariable analysis (adjusted HR=1.35(1.07, 1.71), *P*= .012) (Table 3 and Supplementary Fig 3C). The median OS was decreased from 6.7 (95% CI: 6.2, 7.2) to 5.2 (95% CI: 4.3, 6.8) years with the AA/AG vs. GG rs1077858 genotypes respectively.

When stratified by metastatic disease status (M0 vs. M1), patients carrying the exonic SNP rs12422149 GG genotype had a trend for shorter OS only in the M1 population and at a

borderline significance, whereas this difference was not observed in the M0 patients (adjusted HR for GG: 0.82 (95%CI: 0.58-1.14) for M0; 1.32(0.97-1.80) for M1, *P* interaction= .038). The association of OS with rs1789693 and rs1077858 were similar between M0 and M1 population (*P* interaction >0.9, Table 3 and Supplementary Fig 4). The minor rs1077858 GG genotype portended shorter OS from ADT initiation in both groups (adjusted HR=1.36 (95%CI: 0.93, 1.99) and 1.35 (95%CI: 1.00, 1.82) respectively) (Table 3 and Supplementary Fig 4).

Impact of the Intronic SNP rs1077858 on Expression of SLCO2B1

In our previous study, we showed that different *SLCO2B1* variants corresponding to the exonic SNP rs12422149G>A (Arg312Gln) influenced the efficiency of DHEAS transport into PC cells.[22] Given the fact that the intronic SNP rs1077858 was associated with OS, we studied the possible mechanism by which the SNP might affect OS from ADT initiation. Using 80 normal prostate tissue samples available in our tissue repository of patients known to have PC, we analyzed SLCO2B1 expression (Fig 1A). Results demonstrated that the different rs1077858 SNPs were associated with significantly different SLCO2B1 expression levels (P_{trend}= .019) with the highest SLCO2B1 expression in patients carrying the minor GG genotype. To prove that different rs1077858 SNPs impact the expression of SLCO2B1, we reconstituted the rs1077858 SNP variants using CRISPR in 22RV1 cells to convert the original A allele to the G allele (Supplementary data). The association between different rs1077858 SNPs and SLCO2B1 expression levels was significant (P_{trend}= .0073) and, once again, the GG genotype in 22RV1 cells corresponded to the highest SLCO2B1 expression level (Fig 1B). These results suggest that the association of rs1077858 on OS was possibly through changes in SLCO2B1 expression levels in these patients. As a result, alleles corresponding to higher SLCO2B1 expression may

allow greater DHEAS uptake and thereby induce shorter OS in patients. To evaluate this hypothesis, we examined DHEAS uptake in a LNCaP PC cell line by altering the expression level of SLCO2B1 to mimic different rs1077858 allele conditions. After successfully knocking down or overexpressing SLCO2B1 (Fig 1C), we found that DHEAS uptake activity was dependent on the expression level of SLCO2B1 and that higher expression of SLCO2B1 transported more DHEAS into the cells (Fig 1D).

DISCUSSION

There is great variability in the durability of response to ADT, and multiple mechanisms are likely operative. Persistent androgens, especially adrenal androgen precursors such as DHEAS, play an important role in the evolution of CRPC by continuing to drive the AR signaling pathway.[15,25,26] SLCO2B1 is an active androgen transporter that pumps androgens and androgen precursors into prostate cells, potentially promoting resistance to ADT.[21,22,27] We have found that *SLCO2B1* SNPs are significantly associated with either the efficiency of DHEAS transport or the expression level of SLCO2B1 on prostate cells, which may in part mechanistically determine the variability in outcomes on ADT.

Our initial study revealed that three *SLCO2B1* SNPs significantly influenced TTP on ADT in PC patients with approximately 10 months differences in median TTP.[22] In addition, an independent study of the *SLCO2B1* SNP in a Japanese population also found that rs12422149 associate with TTP during ADT.[23] In the current study, we attempted to further validate those findings in an independent cohort of patients and to better elucidate the mechanism behind these effects. Only the exonic SNP rs12422149 was validated for the association with TTP on ADT in this study, such that PC patients who carry the major GG genotype for rs12422149 may derive

less benefit from ADT. Increased uptake in the poorer risk allele carriers may be associated with greater intracellular androgen stores and thus, is a plausible mechanism of resistance to ADT. In addition, in subset analysis, the association of rs12422149 with TTP on ADT was seen predominantly in M1 patients, further suggesting additional factors yet to be elucidated may participate in resistance in M1 patients compared to the earlier M0 disease state.

The difference in median TTP between the AA and GG rs1077858 genotypes in the validation cohort was 6.8 months (AA: 20.9 months (95% CI: 17.0, 25.1) vs. GG: 14.1 months (95% CI: 9.7, 23.7)), which has a similar trend to that observed in our initial study. This difference did not persist in the multivariable model after adjusting for other clinical factors. False discovery is certainly a possible reason for our inability to validate an association between the two intronic SNPs, rs1789693 and rs1077858, and TTP on ADT in the current study.

To our knowledge, the current study is the first one to demonstrate that an inherited variation in the *SLCO2B1*gene was significantly associated with OS after ADT. While the intronic SNP rs1077858 correlated with differences in TTP in the initial cohort, the current study should be viewed as hypothesis generating. In the current study, it was found to be associated with OS in the larger combined cohort of patients. Importantly, the phenotype of this functional SNP is independent of metastatic disease status at ADT initiation, indicating that the decreased OS cannot be attributed to lead time bias, i.e., starting ADT later when patients have radiographically evident metastases compared to biochemical recurrence only. Taking it a step further mechanistically, we demonstrated that genetic variations in rs1077858 correlate with variable expression levels of SLCO2B1 in patient prostate tissue samples and in a cell line based study. As such, it is plausible that the rs1077858 GG variant with its higher level of SLCO2B1 expression results in enhanced DHEAS uptake and increased activation of the AR signaling

pathway with subsequent shorter OS in patients. It is unclear why we observed an OS benefit but no definite impact on TTP with rs1077858 in the validation cohort. However, there was a median 6 month increase in TTP which was consistent with an improvement in outcomes with this SNP but this observation did not reach statistical significance in the univariate model (TTP on ADT for AA and GG is 20.9 and 14.1 months, respectively. *P*=.383).

One must also question why the only SNP validated to influence TTP on ADT, the exonic rs12422149, was not associated with OS. It is worth mentioning that although not associated with OS, GG genotype of rs12422149 had shorter TTP and OS in M1 patients, which suggests that rs12422149, to some extent, could affect the TTP and OS in a consistent fashion. Since the clinical evidence for a significant association of TTP on ADT with OS has not been established, and the exonic SNP rs12422149 functionally affecting DHEAS uptake efficiency has a significant association with TTP on ADT but not with OS in all patients, we believe that mechanisms other than the expression level of SLCO2B1 may be involved in the association of rs1077858 with OS. For example, variability in DHEAS uptake may be an important driver in earlier stages of metastatic disease but non-androgen pathways may induce resistance in later stages. It is also possible that unidentified regulatory or functional SNPs crosstalk with the known functional SNPs in different disease stages in this study. For example, Hearn et. al. have found that men inheriting the variant *HSD3B1* (1245C) allele that enhances DHT synthesis exhibit resistance to ADT, as manifested by worse clinical outcomes (shorter survival time).[28] Therefore, those unidentified factors, such as SNP in *HSD3B1* (A1245C), need to be considered in future analysis to further characterize the association of SNPs in *SLCO2B1* with TTP and OS. Finally and most plausibly with respect to finding a TTP but not OS difference is heterogeneity

in terms of access and administration of the now 5 therapies approved for CRPC that improve survival including enzalutamide, abiraterone, cabazitaxel, radium-223, and sipuleucel-T.[29-35]

Additional potential confounders of our results include differences between the initial and validation cohorts in terms of patient and disease characteristics at diagnosis and at ADT initiation. Patients from the validation cohort were more likely to have a lower PSA at diagnosis and to have received hormonal therapy as part of local therapy (Supplementary Table 1). Thus, the validation cohort could have been biased toward living longer in one respect but this could be countered by the higher use of prior ADT, which would portend a shorter time on ADT.[3] When prior use of ADT was added to the multivariable model, it was not found to impact the result. Finally, differences in access and administration of subsequent survival improving therapies such as enzalutamide and abiraterone may have also confounded the OS results. Meanwhile, we found that the exonic SNP rs12422149 effect on TTP was not dependent on prior ADT but the effect of the intronic SNP rs1077858 was dependent on prior ADT (Supplementary Table 2). Thus, future analyses should be performed stratifying by disease stage, use of ADT with local therapy, and subsequent therapies for metastatic disease to disclose the unknown factors that affect the different SNP's functions at different stages of the disease and result from prior ADT use.

In conclusion, germline variants within *SLCO2B1* appear to modulate function or expression of SLCO2B1 *in vitro*, which consequently may affect the uptake of DHEAS and impact TTP and OS in PC patients. These findings support that *SLCO2B1* variants are prognostic markers of durability of response to ADT. Further, SLCO2B1 may serve as a target for novel agents and strategies. Ultimately, if prospectively validated, genotyping of *SLCO2B1* for patients may help tailor more effective therapies to the individual patient.

Funding source: The research was funded by the Dana-Farber Prostate Cancer SPORE P50CA090381 and the Department of Defense (DOD - W81XWH-14-1-0515) (Kantoff) and by the Prostate Cancer Foundation Young Investigator Award (Harshman).

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Tables

Table 1. *SLCO2B1* Genotype Distributions and Their Association with TTP on ADT in Validation Cohort

Table 2. Association of *SLCO2B1* Genotype with TTP on ADT in the Combined (Initial plus Validation) Cohort and Stratified by Metastatic Disease Status at ADT Initiation

Table 3. Association of *SLCO2B1* Genotype with OS from the Initiation of ADT in the Combined (Initial plus Validation) Cohort and Stratified by Metastatic Disease Status at ADT Initiation

Figure Legends

Fig 1. Impact of the intronic SNP rs1077858 on the expression of SLCO2B1 which affects the sulfated dehydroepiandrosterone (DHEAS) uptake. (A) Relative SLCO2B1 mRNA levels compared to GAPDH in normal prostate tissue in primary prostate cancer patients. Significant differences were observed among three groups, P_{trend} <0.05. Lines in the figure represent the median with interquartile range. (B) Relative SLCO2B1 mRNA levels compared to GAPDH in 22RV1 cells with different alleles after CRISPR reconstitution. Significant difference were observed among three groups, P_{trend}<0.01. Lines in the figure represent the median with interquartile range. (C) SLCO2B1 mRNA levels in mock transfected LNCaP cells and LNCaP cells transfected with different SLCO2B1 variants. Values represent the fold differences relative to those in mock transfected cells, which were set as 1.0.WT, LNCaP mock transfected with either siRNA control or pCMV6-XL4 vector; siSLCO2B1, LNCaP transiently transfected with siRNA targeting SLCO2B1 (-p represents the SMARTpool siRNA from GE Healthcare; -1, -2 and -3 represent three unique 27mer siRNAs from Origene, respectively. See Supplementary Data for details); oeSLCO2B1, LNCaP transiently transfected with pCMV6-XL4-SLCO2B1. All experiments were repeated in triplex. Statistical analysis (unpaired t-test) was performed by comparing each condition with the WT condition. All mRNA levels were analyzed by quantitative reverse transcriptase polymerase chain reaction and normalized by the expression level of glyceraldehyde 3-phosphate dehydrogenase. (D) DHEAS uptake in LNCaP cells. Cells were incubated with 2.5 μM DHEAS for 10 minutes after cells have been transfected with siRNA or pCMV6-XL4-SLCO2B1 for 3 days. All experiments were repeated in triplex. Statistical analysis (unpaired t-test) was performed by comparing each condition with the WT condition.

Figure 1

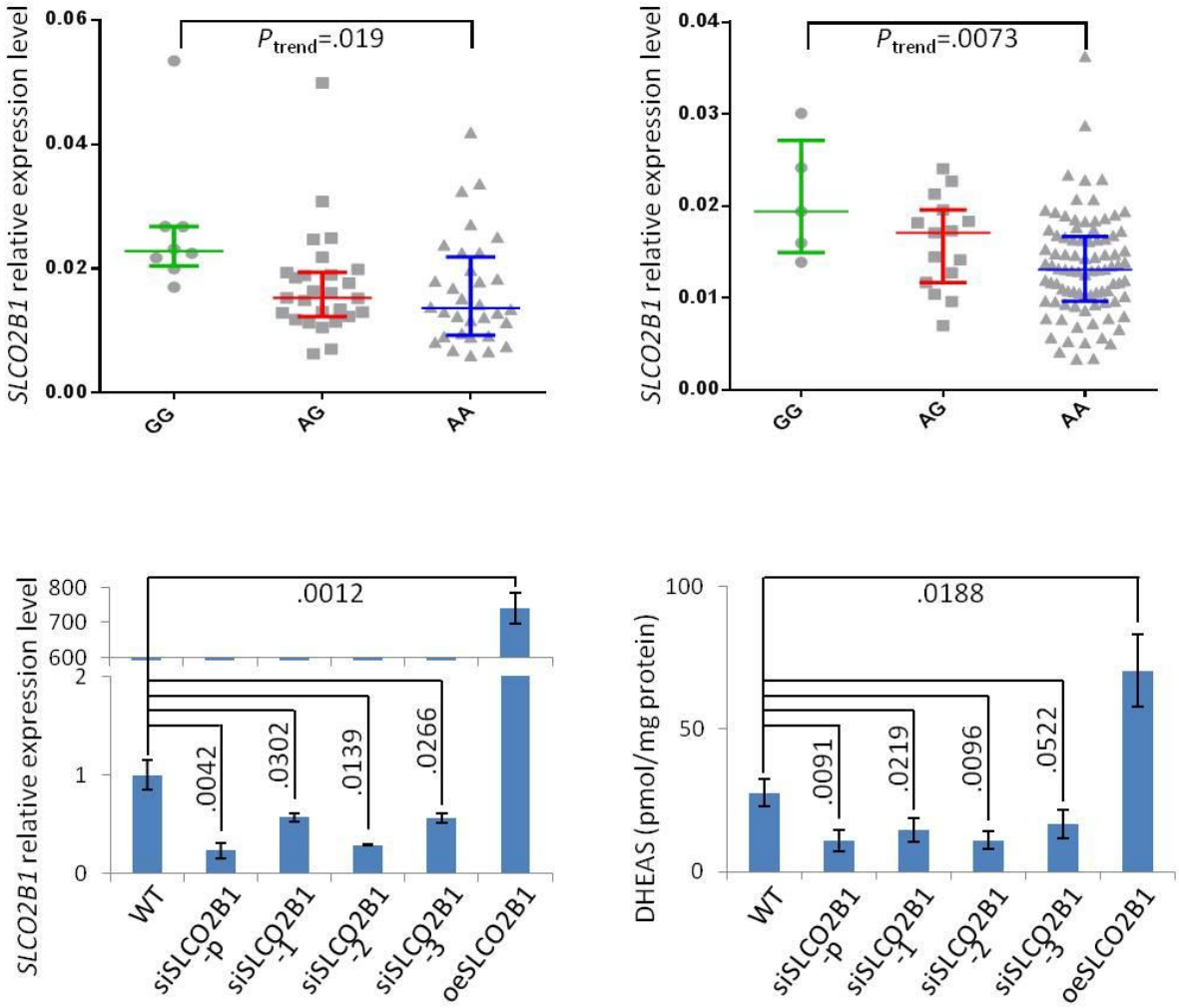

SLCO2B1 relative expression level
0.06
0.04
0.02
0.00
P_{trend}=.019
GG
AG
AA
SLCO2B1 relative expression level
0.04
0.03
0.02
0.01
0.00
P_{trend}=.0073
GG
AG
AA
SLCO2B1 relative expression level
800
700
600
2
1
0
.0012
.0042
.0302
.0139
.0266
WT
siSLCO2B1-p
siSLCO2B1-1
siSLCO2B1-2
siSLCO2B1-3
oeSLCO2B1
DHEAS (pmol/mg protein)
100
50
0
.0188
.0091
.0219
.0096
.0522
WT
siSLCO2B1-p
siSLCO2B1-1
siSLCO2B1-2
siSLCO2B1-3
oeSLCO2B1

Table 1. *SLCO2B1* Genotype Distributions and Their Association with TTP on ADT in Validation Cohort

Genotype	N(%)	Univariate Model			Multivariate Model**	
		Median TTP (95% CI), months	*Log-rank* *P* value	HR (95% CI)	Adjusted HR (95% CI)	*P* value
rs12422149						
AA/AG	110(18)	27.2(18.9,48.9)	0.019	1.00 (reference)	1.00 (reference)	0.049
GG	506(82)	20.0(16.5,23.0)		1.39(1.05,1.80)	1.31(1.00,1.72)	
rs1789693						
AA	238(39)	21.6(17.5,34.1)	0.176	1.00 (reference)	1.00 (reference)	0.409
AT	281(46)	19.4(15.0,23.4)		1.22(0.99,1.51)	1.15(0.93,1.43)	
TT	90(15)	25.2(18.1,38.1)		1.07(0.79,1.45)	1.02(0.75,1.39)	
rs1077858						
AA	288(47)	20.9(17.0,25.1)	0.383	1.00 (reference)	1.00 (reference)	0.880
AG	242(40)	23.0(16.6,30.0)	(0.185)*	0.96(0.77,1.18)	0.98(0.79,1.22)	(0.634)*
GG	81(13)	14.1(9.7,23.7)		1.19(0.88,1.59)	1.06(0.79,1.44)	

*If AA and AG were combined.

** Adjusting for biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with local therapy, metastatic status and PSA at initiation of ADT.

Table 2. Association of *SLCO2B1* Genotype with TTP on ADT in the Combined (Initial plus Validation) Cohort and Stratified by Metastatic Disease Status at ADT Initiation

Genotype	Combined Cohort			By Metastatic disease status**				
				M0		M1		*P* value (interaction)
	N(%)	Adjusted HR (95% CI)*	*P* value	N	Adjusted HR (95% CI)	N	Adjusted HR (95% CI)	
rs12422149								
AA/AG	197(18)	1.00 (reference)	0.003	93	1(reference)	104	1(reference)	0.006
GG	894(82)	1.33(1.10,1.60)		366	0.96(0.72,1.27)	528	1.65(1.28, 2.12)	
rs1789693								
AA	427(39)	1.00 (reference)	0.978	186	1(reference)	241	1(reference)	0.551
AT	486(45)	0.99(0.85,1.16)		200	1.07(0.83,1.37)	286	0.95(0.78, 1.15)	
TT	169(16)	1.02(0.83,1.25)		68	1.18(0.83,1.67)	101	0.94(0.72, 1.21)	
rs1077858								
AA	463(43)	1.00 (reference)	0.075	197	1(reference)	266	1(reference)	0.610
AG	476(44)	1.11(0.95,1.29)	(0.062)*	200	1.21(0.95,1.55)	276	1.05(0.87, 1.27)	
GG	147(14)	1.27(1.03,1.58)		60	1.27(0.88,1.81)	87	1.27(0.98,1.66)	

* Adjusting for biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with local therapy, metastatic status and PSA at initiation of ADT.
** From multivariable Cox regression models, with the interaction of SNP and metastatic disease and simultaneously adjusted for other clinical variables. P value for interaction is to test whether the association of SNP with TTP differs by metastatic disease status.

Table 3. Association of *SLCO2B1* Genotype with OS from the Initiation of ADT in the Combined (Initial plus Validation) Cohort and Stratified by Metastatic Disease Status at ADT Initiation

Genotype	Combined Cohort						By Metastatic Disease Status***				
		Univariate Model			Multivariate Model**		M0		M1		*P* value (inter-action)
	N(%)	Median OS (95% CI), years	Log-rank P value (Bonferroni adjusted)	HR (95% CI)	Adjusted HR (95% CI)	*P* value	N	Adjusted HR (95% CI)	N	Adjusted HR (95% CI)	
rs12422149											
AA/AG	197(18)	7.1(6.0, 8.1)	0.373 (0.999)	1.00(reference)	1.00(reference)	0.506	93	1.00(reference)	104	1.00(reference)	0.038
GG	894(82)	6.4(5.9, 6.9)		1.11(0.89,1.39)	1.08(0.86,1.36)		366	0.82(0.58,1.14)	528	1.32(0.97,1.80)	
rs1789693											
AA	427(39)	6.1(5.2, 6.7)	0.184 (0.552)	1.00(reference)	1.00(reference)	0.044	186	1.00(reference)	241	1.00(reference)	0.999
AT	486(45)	7.2(6.1, 7.7)		0.84(0.70,1.01)	0.81(0.67,0.98)		200	0.81(0.60,1.11)	286	0.81(0.64,1.03)	
TT	169(16)	6.2(5.0, 7.8)		0.89(0.69,1.14)	0.78(0.60,1.00)		68	0.78(0.50,1.21)	101	0.77(0.57,1.06)	
rs1077858											
AA/AG*	939(86)	6.7(6.2, 7.2)	0.009 (0.027)	1.00(reference)	1.00(reference)	0.012	397	1.00(reference)	542	1.00(reference)	0.970
GG	147(14)	5.2(4.3, 6.8)		1.36(1.08,1.71)	1.35(1.07,1.71)		60	1.36(0.93,1.99)	87	1.35(1.00,1.82)	

*Distributions of OS were similar between AA and AG group, thus were combined (median 6.8 and 6.5 years, respectively)

**Adjusting for biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with local therapy, metastatic status, PSA at initiation of ADT and age at ADT initiation.

***From multivariable Cox regression models, with the interaction of SNP and metastatic disease and simultaneously adjusted for other clinical variables. P value for interaction is to test whether the association of SNP with TTP differs by metastatic disease status.

SUPPLEMENTARY DATA

Patients and methods

Briefly, the initial ADT cohort (N=595) was identified in June 2006 and the patients' treatments and outcome data were updated in 2012. Of these, 27 patients were excluded due to insufficient follow-up data after ADT initiation and 77 were excluded because they started ADT prior to 1996. This year was used as a cutoff because patients who had lived that long (i.e., at least 6 yrs from time of ADT initiation) were excellent responders and inclusion would have created the potential for selection bias. Of the remaining 491 patients, 478 were genotyped for the three *SLCO2B1* SNPs. In the validation cohort, 758 patients were identified and clinical data for this analysis was retrieved from the CRIS database in 2013. Of these, 104 patients were excluded due to insufficient follow-up data after ADT initiation and 11 were excluded because they started ADT prior to 1996. Of the remaining 643 patients, 616 were genotyped for the three *SLCO2B1* SNPs.

TTP was defined as two rises in prostate-specific antigen (PSA) (at least 1 week apart) while receiving ADT. The first rise was required to be greater than the nadir PSA value plus 0.02 ng/ml. Initiation of a second treatment for rising PSA before fulfillment of the definition of progression was also considered as a progression event; the date of starting the secondary treatment was denoted as the date of progression. TTP during ADT was defined as the duration of time from ADT initiation to the date of ADT progression or the date of initiation of secondary therapy. Among patients who did not progress, they were censored as of the date of their last known progression-free visit or the date of death

in those who died without progression. OS was defined as the period from ADT initiation to patient death or was censored at date of last follow up.

Statistical Methods

SNPs were analyzed as three distinct genotype groups, with the exception of the exonic SNP rs12422149 analysis wherein the AA and AG were combined because only 1% of the population carries the AA genotype. AA and AG were also combined for the intronic rs1077858 SNP during the analysis of their association with OS since the distributions of OS were similar between the AA and AG groups.

Progression was defined as a minimum of two rises in prostate-specific antigen (PSA), with the date of first rise (nadir + >0.02 ng/ml) as the progression date. TTP during ADT was defined as the duration of time from ADT initiation to the date of ADT progression or the date of initiation of a secondary therapy (for rising PSA before fulfillment of the definition of progression), or was censored at the date of last follow-up visit or PSA value among patients who did not progress. OS was defined as the period from ADT initiation to patient death, or it was censored on the day of last follow up.

The distributions of TTP and OS were estimated using the Kaplan-Meier method, with 95% confidence intervals (CIs). The association between SNPs and TTP was evaluated using the log-rank test or the Wald chi-square test and by multivariable Cox regression model adjusted for known prognostic factors including biopsy Gleason score, type of primary therapy, use of prior ADT in conjunction with local therapy, metastatic status and PSA at ADT initiation. OS was analyzed similarly, but age at ADT initiation was

also included as a covariate in the multivariable models. To estimate whether the association of SNPs with TTP during ADT and OS differed by metastatic disease status at ADT initiation, multivariable Cox regression models were performed with the interaction term of SNP and metastatic disease status, and simultaneously adjusted for the other clinical variables.

For TTP analysis, there was no correction for multiple comparisons since the validation part mainly aimed to reproduce the strength of association previously identified in our initial work where we had applied appropriate correction for multiple comparisons. For the test of OS, Bonferroni procedure was used to correct multiple comparisons.

For *in vitro* cell line studies, data was represented as means ± standard deviation of at least 3 biological repeats. Comparison between two independent groups was performed by an unpaired 2-tailed t test. $P < .05$ (two sided) was considered statistically significant for all analyses.

siRNA

The efficiency of knocking down SLCO2B1 expression was assayed after siRNA transfection for 48 hours by RT-PCR (Forward oligo is GTTTCGGCGAAAGGTCTTAGCAG and reverse oligo is CCATCCTGCTTCTTCGTGGACT, which were purchased from Origene). siSLCO2B1-p was purchased from GE Healthcare as a SMARTpool with 4 target sequences (CAUCCAUGGCUGCGGGCAU; GCCACCAGAUUGCGGGCAU;

UCUCGGAGCCAUACCGCUA; AUAAUGACCUGCUCCGAAA). siSLCO2B1-1, -2, AND -3 were unique 25mer siRNA duplexes and purchased from Origene (-1, AGUCGGGAAUUAUAGAUACAGCUTA; -2, CUACUACAAUAAUGACCUGCUCCGA; -3, GGAUAUGCCACAGGACUUCAAGGCT).

Point mutation at site of rs1077858 by CRISPR

LNCaP (AG genotype for rs1077858) and 22RV1 (AA genotype for rs1077858) cells were chosen to reconstitute rs1077858 GG genotype by CRISPR. [1,2] Briefly, 5 different target oligos (Oligo 1-F, caccgGGCAGGGTCTCGCTCCATTC; Oligo 1-R, aaacGAATGGAGCGAGACCCTGCCc; Oligo 2-F, caccgGCAGGGTCTCGCTCCATTCA; Oligo 2-R, aaacTGAATGGAGCGAGACCCTGCc; Oligo 3-F, caccgGAGGTTACGTAGTCCCTGAA; Oligo 3-R, aaacTTCAGGGACTACGTAACCTCc; Oligo 4-F, caccgTTGTATCTCTCACATTAGAC; Oligo 4-R, aaacGTCTAATGTGAGAGATACAAc; Oligo 5-F, caccgCGAGACCCTGCCTTGTCTGA; Oligo 5-R, aaacTCAGACAAGGCAGGGTCTCGc) were synthesized and cloned into lentiCRISPRv2 one vector system, respectively. A single strand DNA oligo of 101 nucleotides was synthesized as the donor template, which have 50bp of homology arms on each side flanking the change site for rs1077858 A→G. The donor DNA sequence is

AAAGGCCCCTGTCAGATGGGGGAGACAGTCTCTGAGCTGGGGGAAGCCCCT GTCTAATGTGAGAGATACAATCTCTCTGATGGAGGTTAC**G**TAGTCCCTGAAT GGAGCGAGACCCTGCCTTGTCTGATGGAAGAGACCTGACCTCTGTGATGGAG AAGGCCCCTGTCTGATAAGGGAGACG. 2µl of 10µM donor oligo was used for each well of 24-well plate to co-deliver into the cells with 5 constructs. After puromycin selection, cells derived from single colony were assayed for point mutation (Taqman SNP assay) and SLCO2B1 mRNA levels (RT-PCR).

We have assayed 22 single colonies for LNCaP cells and 109 single colonies for 22RV1 cells. Unfortunately, we did not detect any A → G mutation for LNCaP cells. For 22RV1 cells, 89 single colonies remained AA genotype, 15 single colonies are AG, and 5 of them are GG.

1. Ran FA, Hsu PD, Lin CY, et al: Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380-9, 2013

2. Sanjana NE, Shalem O, Zhang F: Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 11:783-4, 2014

Supplementary Table 1. Patient Characteristics and Outcomes in Initial, Validation and Combined Cohorts

	Initial Cohort (N=478)		Validation Cohort (N=616)		Combined Cohorts (N=1094)	
	N	%	N	%	N	%
At diagnosis						
PSA, ng/mL	412	*14*	537	*9.9*	949	*11.6*
Median, IQR		*(7.0,46.0)*		*(5.7,24.8)*		*(6.1,31.8)*
Age, year	458	*62*	580	*61*	1038	*61*
Median, IQR		*(56,67)*		*(56,67)*		*(56,67)*
Clinical T stage						
Tx/Unknown	194	*40.6*	99	*16.1*	293	*26.8*
T1	122	*25.5*	379	*61.5*	501	*45.8*
T2	137	*28.7*	110	*17.9*	247	*22.6*
T3-4	25	*5.2*	28	*4.5*	53	*4.8*
Clinical N stage						
Nx/Unknown	278	*58.2*	349	*56.6*	627	*57.3*
N0	166	*34.7*	213	*34.6*	379	*34.6*
N1	34	*7.1*	54	*8.8*	88	*8.0*
Clinical M stage						
Mx/Unknown	217	*45.4*	316	*51.3*	533	*48.7*
M0	181	*37.9*	208	*33.8*	389	*35.6*
M1	80	*16.7*	92	*14.9*	172	*15.7*

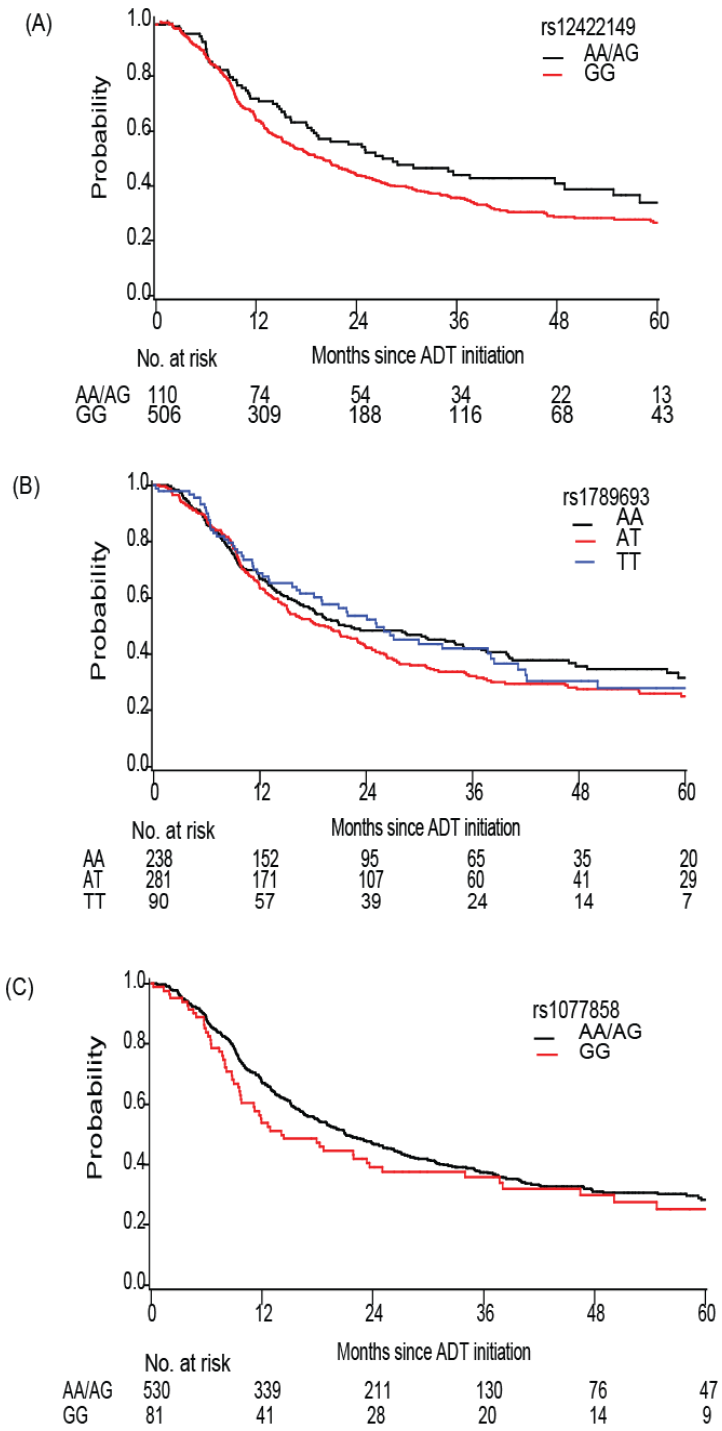
	Initial Cohort (N=478)		Validation Cohort (N=616)		Combined Cohorts (N=1094)	
	N	%	N	%	N	%
Biopsy Gleason score						
6 or less	77	*16.1*	81	*13.1*	158	*14.4*
7	153	*32.0*	198	*32.1*	351	*32.1*
8 or more	171	*35.8*	274	*44.5*	445	*40.7*
Unknown	77	*16.1*	63	*10.2*	140	*12.8*
Type of local therapy						
RP +/-RT	187	*39.1*	264	*42.9*	451	*41.2*
RT only/other	143	*29.9*	201	*32.6*	344	*31.4*
None	148	*31.0*	151	*24.5*	299	*27.3*
Use of ADT as part of local therapy	84	*17.6*	204	*33.1*	288	*26.3*
At ADT initiation						
PSA, ng/mL	380	*14.9*	586	*10.1*	966	*11.9*
Median, IQR		*(5.2,68.4)*		*(4.1,36.5)*		*(4.5,46.8)*
Presence of metastases	274	*57.3*	360	*58.4*	634	*58.0*
Received Anti-androgen during ADT	333	*69.7*	414	*67.2*	747	*68.3*
Received intermittent ADT	90	*18.8*	221	*35.9*	311	*28.4*

Abbreviations: ADT, androgen deprivation therapy; RP, radical prostatectomy; RT, radiation therapy; M, metastatic disease

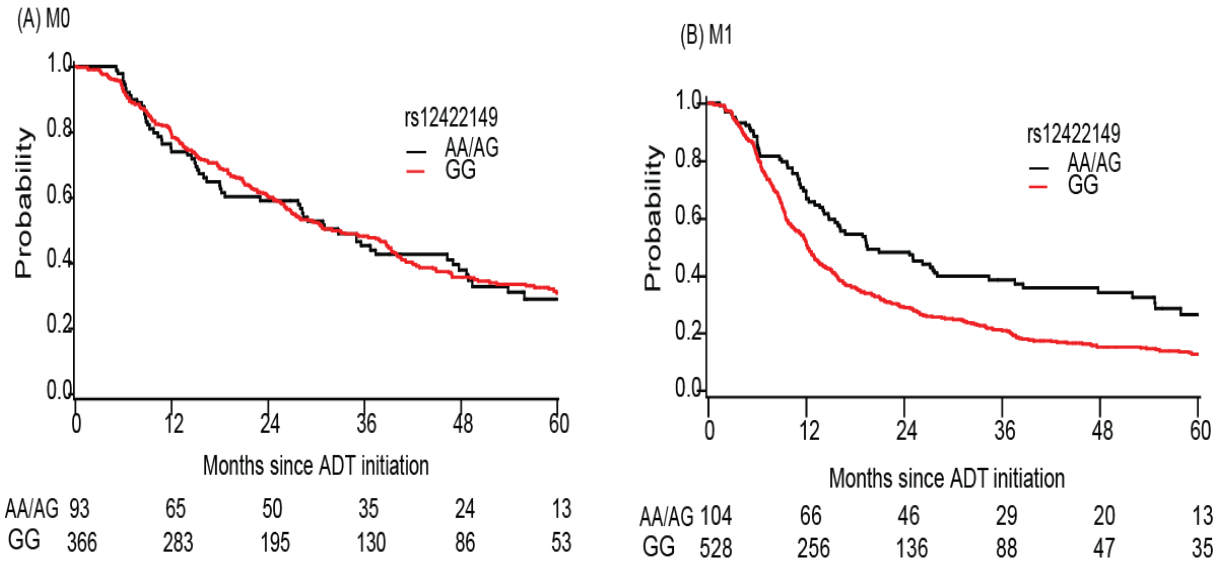
Supplementary Table 2. Association of SLCO2B1 Genotype with TTP on ADT in Combined (Initial plus Validation) Cohort Stratified by Status of Prior Hormone Therapy

	No prior hormone		With prior hormone		
	N	Adjusted HR (95% CI)	N	Adjusted HR (95% CI)	*P* value (interaction)
rs12422149					
AA/AG	144	1(reference)	53	1(reference)	0.642
GG	659	1.29 (1.04,1.60)	235	1.44 (0.97,2.13)	
rs1789693					
AA	318	1(reference)	109	1(reference)	0.517
AT	358	0.95 (0.79, 1.13)	128	1.17 (0.85, 1.59)	
TT	123	0.99 (0.78, 1.26)	46	1.09 (0.72, 1.67)	
rs1077858					
AA/AG	684	1(reference)	255	1(reference)	0.104
GG	116	1.32 (1.06,1.65)	31	0.87 (0.55,1.37)	

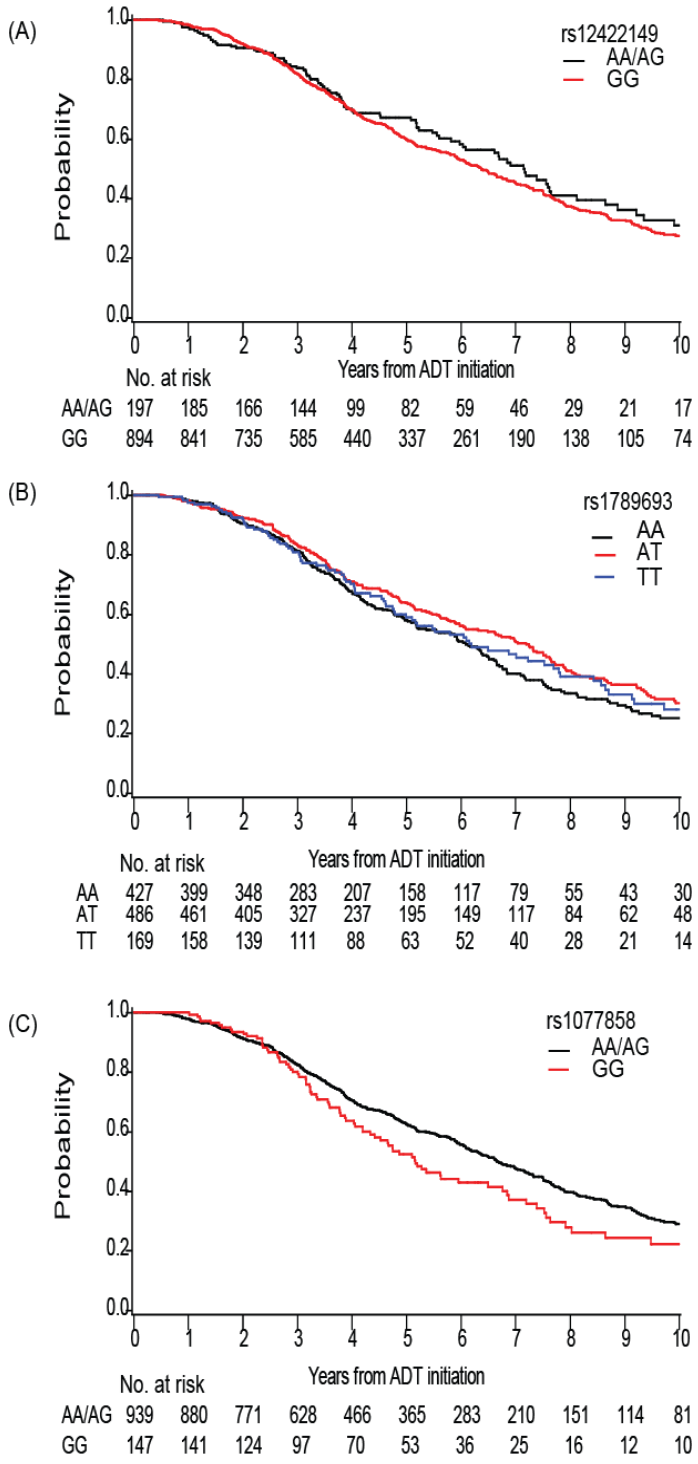
Supplementary Fig 1. Kaplan-Meier curves of time to progression during ADT in the validation cohort, according to *SLCO2B1* genotypes (A) rs12422149, (B) rs1789693, (C) rs1077858.



Supplementary Fig 2. Kaplan-Meier curves of time to progression during ADT in all cohorts (initial plus validation cohort) according to rs12422149 genotypes as stratified by metastatic status (A) M0 versus (B) M1 at ADT initiation.



Supplementary Fig 3. Kaplan-Meier curves of overall survival from ADT initiation in all patients (initial plus validation cohort), according to *SLCO2B1* genotypes (A) rs12422149, (B) rs1789693, (C) rs1077858.



Supplementary Fig 4. Kaplan-Meier curves of overall survival from ADT initiation in all cohorts, according to *SLCO2B1* genotypes at rs12422149 (A and B) and rs1077858 (C and D), stratified by metastatic status at ADT initiation.